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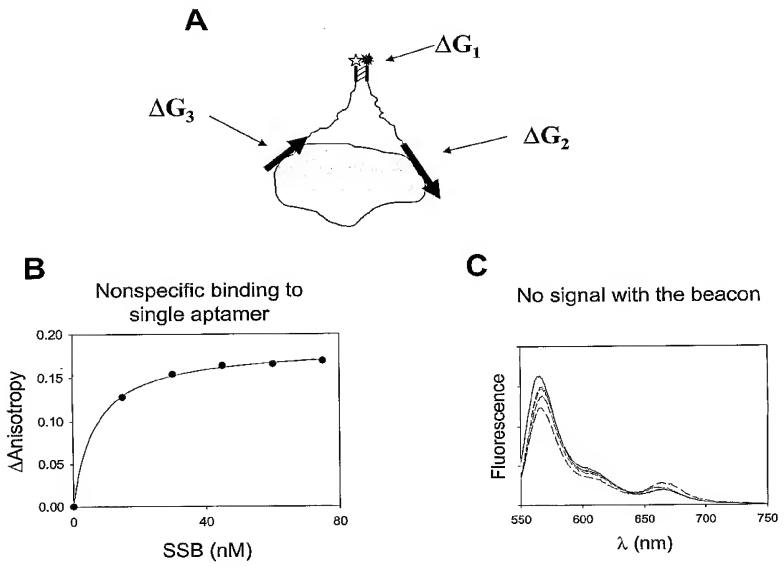

(57) Abstract: Described are compositions and methods that are useful in the identification and quantification of any polypeptide or macromolecular complex using a set of co-aptamer constructs. Aptamer constructs are constructed that bind to unique epitopes of a polypeptide of macromolecular construct. Those aptamer constructs contain an epitope binding site, a co-aptamer binding site, and a detectable label. In the presence of the cognate polypeptide, analyte-polypeptide complex, or other macromolecular complex, the co-aptamers associate with one another to produce a detectable signal. The co-aptamer constructs may be joined by a linker to produce a bivalent aptamer construct.

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BIOSENSORS FOR DETECTING MACROMOLECULES AND OTHER ANALYTES

PARENT CASE TEXT

[0001] This patent application claims priority to U.S. Provisional Patent Application No. 60/529,076, which was filed on December 12, 2003.

GOVERNMENTAL SUPPORT

[0002] This work was supported by the U.S. Department of Health and Human Services/ National Institutes of Health grant number CA94356. The U.S. Government has certain rights in this invention.

SEQUENCE LISTING

[0003] A paper copy of the sequence listing and a computer readable form of the same sequence listing are appended below and herein incorporated by reference. The information recorded in computer readable form is identical to the written sequence listing, according to 37 C.F.R. 1.821 (f).

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0004] The invention relates to kits, molecular beacons, and methods for detecting any polypeptide, analyte, macromolecular complex, or combination thereof. The invention relates to biomedical research tools and diagnostic kits.

2. Description of the Related Art

[0005] The detection, identification and quantification of specific molecules in our environment, food supply, water supply and biological samples (blood, cerebral spinal fluid, urine, et cetera) can be very complex, expensive and time consuming. Those methods include gas chromatography, mass spectroscopy, DNA sequencing, immunoassays, cell-based assays, biomolecular blots and gels, and myriad other multi-step chemical and physical assays.

[0006] There continues to be a high demand for convenient methodologies for detecting and measuring the levels of specific proteins in biological and environmental samples. Detecting and measuring levels of proteins is one of the most fundamental and most often performed methodologies in biomedical research. While antibody-based protein detection methodologies are enormously useful in research and medical diagnostics, they are not well adapted to rapid, high-throughput parallel protein detection.

[0007] Previously, the inventor had developed a fluorescent sensor methodology for detecting a specific subclass of proteins, i.e., sequence-specific DNA binding protein (Heyduk, T.; Heyduk, E. *Nature Biotechnology* **2002**, 20, 171-176; Heyduk, E.; Knoll, E.; Heyduk, T. *Analyt. Biochem.* **2003**, 316, 1-10; U.S. Patent No. 6,544,746 and copending patent applications number 10/062,064, PCT/US02/24822 and PCT/US03/02157, which are incorporated herein by reference). This methodology is based on splitting the DNA binding site of proteins into two DNA "half-sites". Each of the resulting "half-sites" contains a short complementary single-stranded region of the length designed to introduce some propensity for the two DNA "half-sites" to associate recreating the duplex containing the fully functional protein binding site. This propensity is designed to be low such that in the absence of the protein only a small fraction of DNA half-sites will associate. When the protein is present in the reaction mixture, it will bind only to the duplex containing fully functional binding site. This selective binding will drive association of DNA half-sites and this protein-dependent association can be used to generate a spectroscopic signal reporting the presence of the target protein. The term "molecular beacons" is used in the art to describe the above assay to emphasize that selective recognition and generation of the signal reporting the recognition occur in this assay simultaneously. Molecular beacons for DNA binding proteins have been developed for several proteins illustrating their general applicability (Heyduk, T.; Heyduk, E. *Nature Biotechnology* **2002**, 20, 171-176, which is herein incorporated by reference). Their physical mechanism of action has been established and they have been also used as a platform for the assay detecting the presence of ligands binding to DNA binding proteins (Heyduk, E.; Knoll, E.; Heyduk, T. *Analyt. Biochem.* **2003**, 316, 1-10; Knoll, E.; Heyduk, T. *Analyt. Chem.* **2004**, 76, 1156-1164; Heyduk, E.; Fei, Y.; Heyduk, T. *Combinatorial Chemistry and High-throughput Screening* **2003**, 6, 183-194, which are incorporated herein by reference.) While already very useful, this assay is limited to proteins that exhibit natural DNA binding activity.

Aptamers as "Molecular Beacons"

[0008] Development of convenient, specific, sensitive high-throughput assays for detecting proteins remains an extremely important goal. Such assays find applications in research, drug

discovery and medical diagnosis. Antibodies recognizing target protein are the centerpiece of the great majority of protein detection assays so far. Development of *in vitro* methods for selecting aptamers recognizing target proteins from a population of random sequence nucleic acids provided the first real alternative to antibodies. One of the potentially important advantages of aptamers is that they are made of easy to propagate and synthesize oligonucleotides. Additionally, standard nucleic acid chemistry procedures can be used to engineer aptamers to contain reporter groups such as, for example, fluorescence probes. Thus, it is no wonder that there is a significant interest to utilize aptamers in various formats of protein detection assays. One of the most promising routes is the development of aptamer-based sensors combining recognition of the target protein with generation of optical signal reporting the presence of the protein.

[0009] There are several published reports that document ingenious designs of aptamer-based "molecular beacons" which produced fluorescence signal upon binding to a specific target protein. All of these designs rely on target protein-induced conformational transition in the aptamer to generate fluorescence signal change. Yamomoto and Kumar (*Genes to Cells* **2000**, 5, 389-396) described molecular beacon aptamer that produced an increase of fluorescence upon recognition of HIV Tat protein. Fluorescence signal was generated due to a change of proximity of fluorophore-quencher pair resulting from Tat protein-induced transition between hairpin and duplex forms of the aptamer. Hamaguchi et al. (*Analyt. Biochem.* **2001**, 294, 126-131) described molecular beacon aptamer that produced an increase of fluorescence upon recognition of thrombin. In the absence of the target protein, the beacon was designed to form a stem-loop structure bringing fluorophore and the quencher to close proximity. In the presence of the protein, the beacon was forced to ligand-binding conformation resulting in increased separation between fluorophore and the quencher and increased fluorescence signal. Li et al. (*Biochem. Biophys. Res. Commun.* **2002**, 292, 31-40) described molecular beacon aptamer, which underwent transition from loose random coil to a compact unimolecular quadruplex in the presence of a target protein. This protein-induced change in aptamer conformation resulted in a change of proximity between fluorescence probes attached to the ends of the aptamer generating a fluorescence signal change. Analogous approach was used by Fang et al. (*ChemBioChem.* **2003**, 4, 829-834) to design molecular beacon aptamer recognizing PDGF. These examples illustrate the great potential of aptamers for designing sensors, which could transduce the presence of the protein into an optical signal.

SUMMARY OF THE INVENTION

[00010] The inventor has successfully generalized the sensor design applied previously for detecting sequence-specific polynucleotide binding to proteins and other analytes that lack

natural nucleic acid binding activity, to greatly expand the applicability of these sensors. Briefly, natural DNA sequences recognizing sequence specific DNA binding proteins were replaced with nucleic acids that bind a particular target protein obtained by *in vitro* selection from a pool of random sequences. It has been well established that nucleic acid (DNA or RNA) aptamers capable of specific binding to proteins lacking natural DNA binding activity can be produced by *in vitro* SELEX (**s**ystematic **e**volution of **l**igands by **ex**ponential enrichment) procedure (Tuerk, C.; Gold, L. *Science* **1990**, 249, 505-510; Gold, L.; Polisky, B.; Uhlenbeck, O.; Yarus, M. *Ann. Rev. Biochem.* **1995**, 64, 763-797; Wilson, D.S.; Szostak, J.W. *Ann. Rev. Biochem.* **1999**, 68, 611-647). SELEX involves selection of nucleic acid sequences binding a specific target from a pool of random DNA (or RNA) sequences by cycles of binding, washing out unbound sequences, and PCR amplification of target-bound sequences. Numerous examples of successful selection of aptamers specifically binding various proteins as well as other target molecules (Turek 1990, Polisky 1995 and Wilson 1999) provide a strong indication that producing aptamers to a large number of naturally occurring proteins is possible.

[0001 1] The inventors have developed compositions and methods which further enable the application of the proximity-based assay described in copending patent application 10/062,064 (which is incorporated herein by reference) to extend beyond nucleic acid binding factors, their ligands and coregulators to include any polypeptide (including prions or other misfolded proteins), analyte, small molecule ligand or macromolecular complex. The invention is directed to the use of a set of labeled aptamers that contain short (preferably about 5-7 nucleotides) complementary single stranded polynucleotide sequences at the distal tip of each aptamer (called "signaling oligos"). Each aptamer of the set of aptamers binds to a specific and different epitope of a polypeptide or macromolecular complex, i.e., a first aptamer of the set binds to a first epitope of the polypeptide or macromolecular complex, and a second aptamer of the set binds to a second epitope of the polypeptide or macromolecular complex. In the presence of the polypeptide or macromolecular complex, the first aptamer binds to the first epitope and the second aptamer binds to the second epitope such that the short complementary single stranded polynucleotide sequences at the distal tip of each aptamer can stably associate with each other. Upon the stable association of the short single stranded polynucleotide sequence at the distal tip of the first aptamer with the short single stranded polynucleotide sequence at the distal tip of the second aptamer, a label on the first aptamer is brought into proximity to a label on the second aptamer to produce a measurable signal. In other words, two or more novel nucleic acid half sites that bind to any polypeptide or macromolecular complex may be made and used in a proximity-based assay to detect any

polypeptide or macromolecular complex. The set of aptamers may be joined by a flexible linker to form a bivalent aptamer construct.

[00012] One embodiment of the invention is a method comprising making a first aptamer and a second aptamer, which bind to a first epitope and a second epitope respectively of a polypeptide or biomolecular macromolecular complex. The aptamers may be made using in vitro selection, such as systematic evolution of ligands by exponential enrichment (a.k.a. SELEX; see Klug, S.; Famulok, M., All you wanted to know about SELEX. *Mol. Biol. Reports* **1994**, *20*, 97-107, which is incorporated herein by reference.) Alternatively, one or more pre-existing aptamer or naturally occurring cognate nucleic acid sequence may be modified to contain a label and a short single stranded polynucleotide sequence at the distal tip and used to detect a polypeptide, analyte or macromolecular complex.

[00013] In another embodiment, the invention is drawn to bivalent aptamers, which comprise a first aptamer construct, which recognizes a first epitope of a polypeptide or macromolecular complex and contains a first signaling oligo, and a second aptamer construct, which recognizes a first epitope of a polypeptide or macromolecular complex and contains a first signaling oligo, wherein the first aptamer construct and the second aptamer construct are linked together via a flexible linker. In a preferred embodiment, the first aptamer construct contains a first label and the second aptamer construct contains a second label for the purpose of detection.

[00014] It is envisioned that the bivalent aptamer constructs can be useful in myriad applications beyond the detection of molecules and macromolecular complexes. The bivalent aptamers may be used in much the same way as antibodies, e.g., to detect molecules and complexes, to purify molecules and complexes, to block epitopes and antigens, to facilitate immune responses (both innate and specific) in an organism, to treat diseases and to confer passive immunity to a subject. The preferred subject is a human, a farm animal or a companion animal.

[00015] It is further envisioned that the bivalent aptamers can be used as therapeutic compositions to block molecular interactions in a cell or tissue, or to facilitate molecular interactions in a cell or tissue to effect a desired therapeutic outcome in a patient. Preferred patients include humans, farm animals and companion animals.

[00016] It is further envisioned that the aptamer constructs or bivalent aptamer constructs can be used in medical or veterinary diagnostics or in pharmaceutical screens to help identify potential safe and effective pharmaceutical products.

BRIEF DESCRIPTION OF THE DRAWINGS

[00017] Fig. 1. Overall design of molecular beacons for detecting proteins. (A) Variant of the design for proteins lacking natural DNA binding activity. The beacon in this case will be composed of two aptamers developed to recognize two different epitopes of the protein. (B) Variant of the design for protein exhibiting natural DNA binding activity. The beacon in this case will be composed of a short DNA duplex containing the DNA sequence corresponding to the protein binding site and DNA (RNA) aptamer developed to recognize a different epitope of the protein.

[00018] Fig. 2. Methods for preparing co-aptamers directed to an epitope distinct from the binding site of the first aptamer (A) or distinct from the binding site of nucleic acid containing the natural binding site of the protein (B).

[00019] Fig. 3. Comparison of the design of molecular beacons for DNA binding proteins (A) and molecular beacons for detecting proteins based on aptamers directed to two different epitopes of the protein (B).

[00020] Fig. 4. Aptamer constructs containing aptamers binding thrombin at fibrinogen exosite (60-18 [29] and at heparin exosite (G15D).

[00021] Fig. 5. Binding of fluorescein-labeled aptamers to thrombin. (A) Binding of 60-18 [29] aptamer (THR1) (50 nM) detected by fluorescence polarization; (B) Binding of G15D aptamer (THR2) (50 nM) detected by change in fluorescence intensity; (C) Quantitative equilibrium titration of fluorescein-labeled G15D aptamer (THR2) (20 nM) with thrombin. Solid line represents nonlinear fit of experimental data to an equation describing formation of 1:1 complex between the aptamer and thrombin; (D) Quantitative equilibrium titration of fluorescein-labeled G15D aptamer (THR2) (20 nM) with thrombin in the presence of ten fold excess of unlabeled 60-18 [29] aptamer (THR3). Solid line represents nonlinear fit of experimental data to an equation describing formation of 1:1 complex between the aptamer and thrombin

[00022] Fig. 6. Illustration of the competition between thrombin aptamer constructs and fluorescein-labeled G15D aptamer (THR2) for binding to thrombin. Fluorescence spectra of 50 nM fluorescein-labeled G15D (THR2) with and without thrombin in the absence of competitor (A), in the presence of 150 nM THR3 (B), in the presence of 150 nM THR4 (C), and in the presence of 150 nM THR7 (D).

[00023] Fig. 7. Summary of experiments probing competition between thrombin aptamer constructs and fluorescein-labeled G15D aptamer (THR2) for binding to thrombin. Fluorescence intensity of fluorescein-labeled G15D aptamer (THR2) (50 nM) in the absence

and the presence of the competitor (250 nM) was used to determine % of THR2 bound in the presence of the competitor. Thrombin concentration was 75 nM. The values of dissociation constants shown in the figure were calculated from a separate experiment in which 200 nM fluorescein-labeled G15D aptamer (THR2), 200 nM competitor and 150 nM thrombin were used.

[00024] Fig. 8. The effect of 60-18 [29] aptamer (THR3) on the competition between fluorescein-labeled G15D aptamer (THR2) and THR5 construct for binding to thrombin. Fluorescence spectra of 200 nM fluorescein-labeled G15D (THR2) with and without thrombin (150 nM) in the absence of the competitor (A), in the presence of 1000 nM THR3 and 200 nM THR5 (B), in the presence of 1000 nM THR3 (C), and in the presence of 200 nM THR5 (D).

[00025] Fig. 9. Binding of THR7 aptamer construct to thrombin detected by gel electrophoresis mobility shift assay. Samples of 417 nM THR7 were incubated with various amounts of thrombin (0 to 833 nM) and after 15 min incubation were loaded on a native 10% polyacrylamide gel. (A) Image of the gel stained with Sybr Green. (B) Intensity of the band corresponding to THR7-thrombin complex as a function of thrombin concentration

[00026] Fig. 10. Family of bivalent thrombin aptamer constructs in which G15D and 60-18 [29] aptamers were connected to a 20 bp DNA duplex by a 9-27 nt long poly T linker.

[00027] Fig.11. Binding of thrombin to bivalent aptamer constructs (33 nM each) illustrated in Fig. 8 detected by electrophoretic mobility shift assay (EMSA). Asterisk marks the lane best illustrating preferential binding of thrombin to constructs with 27 and 17 nt poly T linker over the constructs with 9 nt poly T linker. Thrombin concentration was varied from 0 to 400 nM.

[00028] Fig. 12. Thrombin beacon design using G15D and 60-18 [29] aptamers connected to 9 bp fluorophore (or quencher)-labeled "signaling" duplex through 17 nt poly T linker. (A) Nucleotide sequence of the fluorescein-labeled G15D construct (THR9) and dabcyl-labeled 60-18 [29] construct (THR8). (B) Mechanism of signaling by thrombin beacon. (C) Fluorescence signal change detected upon addition of thrombin to the thrombin beacon. For comparison, titration of the fluorescein-labeled G15D construct (THR9) with thrombin in the absence of dabcyl-labeled 60-18 [29] construct (THR8) is also shown (donor only curve).

[00029] Fig. 13. Thrombin beacon design using G15D and 60-18 [29] aptamers connected to 9 bp fluorophore (or quencher)-labeled "signaling" duplex through a linker containing 5 Spacer18 units. (A) Nucleotide sequence of the fluorescein-labeled G15D construct (THR21) and dabcyl-labeled 60-18 [29] construct (THR20). (B) Mechanism of signaling by thrombin beacon. (C) Fluorescence signal change detected upon addition of thrombin to the thrombin beacon. For comparison, titration of the fluorescein-labeled G15D construct (THR21) with thrombin in the absence of dabcyl-labeled 60-18 [29] construct (THR20) is also shown (donor

only curve). Inset shows fluorescence emission spectra recorded at various concentrations of thrombin corresponding to data points in the main graph.

[00030] Fig. 14. Binding of thrombin to the beacon illustrated in Fig. 13 (THR20/THR21) detected by gel electrophoresis mobility shift assay. The gel was imaged for fluorescein emission (i.e. only THR21 component of the beacon is visible).

[00031] Fig. 15. (A) Sensitivity of thrombin detection at two different concentrations of the beacon. Red circles: 50 nM THR21 and 95 nM THR20. Blue circles: 5 nM THR21 and 9.5 nM THR20. (B) Specificity of the beacon for thrombin. 50 nM THR21 and 95 nM THR20 were titrated with thrombin (red circles) and trypsin (blue circles).

[00032] Fig. 16. Reversal of thrombin beacon signal by competitor aptamer constructs. Fluorescence intensity of 50 nM THR21, 95 nM THR20, and 100 nM thrombin was measured at increasing concentrations of competitor DNA's. The data are plotted as a relative fluorescence increase with respect to a signal (F_o) of a starting beacon and thrombin mixture. Open blue squares: THR7; filled black circles: THR14/THR15; filled red squares: THR16/THR17; filled blue triangles: THR18/THR19; open magenta triangles: THR3; green filled inverted triangles: THR4; open black triangles: nonspecific single stranded DNA.

[00033] Figure 17 depicts molecular beacons for detecting proteins. Comparison of the design of molecular beacons for DNA binding proteins (A) and molecular beacons for detecting proteins based on aptamers directed to two different epitopes of the protein (B).

[00034] Figure 18 depicts the binding of aptamer constructs to thrombin. (A) Binding of G15D aptamer (THR2) (50 nM) detected by change in fluorescence intensity of 5' fluorescein moiety. Solid line represents the best fit of the experimental data to a simple 1:1 binding isotherm. (B) Binding of G15D aptamer (THR2) in the presence of 10x excess of unlabeled 60-18 [29] aptamer. Solid line represents the best fit of the experimental data to a simple 1:1 binding isotherm. (C) Summary of experiments probing competition between thrombin aptamer constructs and fluorescein-labeled G15D aptamer (THR2). Fluorescence intensity of THR2 (200 nM) was used to determine % THR2 bound in the presence of competitor (200 nM). Thrombin was 150 nM. The labels above each bar indicate relative affinity (expressed as fold increase of affinity constant) of the competitor compared to the affinity of THR2 aptamer. (D) Binding of THR7 aptamer construct to thrombin detected by gel electrophoresis mobility shift assay. Intensity of the band corresponding to THR7-thrombin complex is plotted as a function of thrombin concentration. Inset: Image of the gel stained with Sybr Green. Fluorescence change (%) was calculated as $100^* (I-I_o)/I_o$ where I and I_o correspond to dilution-corrected fluorescence emission intensity observed in the presence and absence of a given thrombin concentration, respectively.

[00035] Figure 19 depicts a thrombin beacon design. G15D and 60-18 [29] aptamers were connected to 7 bp fluorophore (or quencher)-labeled "signaling" duplex through a linker containing 5 Spacer18 units. (A) Nucleotide sequence of the fluorescein-labeled G15D construct (THR21) and dabcyl-labeled 60-18 [29] construct (THR20). X corresponds to Spacer 18 moiety. (B) Mechanism of signaling by thrombin beacon. (C) Fluorescence signal change detected upon addition of thrombin to the thrombin beacon. For comparison, titration of the fluorescein-labeled G15D construct (THR21) with thrombin in the absence of dabcyl-labeled 60-18 [29] construct (THR20) is also shown (donor only curve). Signal change (%) was calculated as 100* (I_o -I)/I_o where I and I_o correspond to dilution-corrected fluorescence emission intensity observed in the presence and absence of a given thrombin concentration, respectively. Inset shows fluorescence emission spectra recorded at various concentrations of thrombin corresponding to data points in the main graph.

[00036] Figure 20 depicts variants of thrombin beacon with various combinations of donor-acceptor fluorophores. (A) fluorescein-dabcyl; (B) fluorescein-Texas Red; (C) fluorescein-Cy5, (D) Cy3-Cy5. Emission spectra of the beacon in the absence (black line) and presence (red line) of thrombin are shown. Insets show false color images of microplate wells containing corresponding beacon and indicated concentrations of thrombin. The images were obtained on Bio-Rad Molecular Imager FX using the following excitation-emission settings: (A) 488 nm laser – 530 nm bandpass filter; (B) 488 nm laser – 640 nm bandpass filter; (C) 488 nm laser – 695 nm bandpass filter; (D) 532 nm laser – 695 nm bandpass filter. Fluorescence is in arbitrary units (corrected for instrument response) and is plotted in a linear scale.

[00037] Figure 21 depicts response curves for the beacon with various combinations of donor-acceptor pairs. (A) fluorescein-dabcyl, (B) fluorescein-Texas Red, (C) Cy3-Cy5, (D) fluorescein-Cy5, (E) europium chelate-Cy5, (F) Fold signal change observed for indicated donor-acceptor pair at saturating thrombin concentration. Insets show expanded view of data points at low thrombin concentrations. In all experiments 5 nM donor-labeled and 5.5 nM acceptor-labeled aptamer constructs were used. Signal change (fold) was calculated as I/I_o where I and I_o correspond to dilution-corrected acceptor fluorescence emission intensity (measured with donor excitation) observed in the presence and absence of a given thrombin concentration, respectively. Buffer background was subtracted from I and I_o before calculating signal change.

[00038] Figure 22 depicts the dependence of the sensitivity of the thrombin beacon on a donor-acceptor pair. Response of 10 nM donor-labeled and 11 nM acceptor-labeled beacon was determined at low thrombin concentrations using beacon labeled with fluorescein-dabcyl pair (triangles), fluorescein-Texas Red pair (inverted triangles), and fluorescein-Cy5 pair (circles).

Averages and standard deviations of four independent experiments are shown. Signal change (fold) was calculated as in Fig. 5.

[00039] Figure 23 depicts the reproducibility and stability of thrombin beacon. (A) Five independent determinations of beacon signal at four different thrombin concentrations were performed. Data shown represent mean +/- standard deviation. (B) Thrombin beacon signal at four thrombin concentrations was monitored over time up to 24 hours. Data shown represent mean +/- standard deviation of 5 independent measurements. Beacon containing 5 nM fluorescein-labeled aptamer (THR21) and 5.5 nM Texas Red-labeled aptamer (THR27) was used in this experiment. Signal change (fold) was calculated as in Fig. 5 except that buffer background was not substracted.

[00040] Figure 24 shows the determination of Z'-factor for thrombin beacon. Panel in the middle of the plot shows the false color image of wells of the microplate corresponding to the experiment shown in a graph (the upper half of wells are + thrombin, the lower half of the wells is – thrombin. Beacon containing with 5 nM fluorescein-labeled aptamer (THR21) and 5.5 nM Texas Red-labeled aptamer (THR27) was used in this experiment. Signal corresponds to a ratio of acceptor to donor emission (in arbitrary units) measured with donor excitation.

[00041] Figure 25 depicts the detection of thrombin in complex mixtures. (A) Response of thrombin beacon at 1 nM thrombin concentration in the absence and presence of the excess of unrelated proteins. The data shown are averages and standard deviation of 4 independent experiments. (B) Detection of thrombin in HeLa extract "spiked" with various amounts of thrombin. Data shown are averages and standard deviation from 3 independent measurements. Concentrations of thrombin in cell extract were: 1.88 nM (light grey bars); 3.75 nM (dark grey bars); 7.5 nM (black bars). Signal for beacon mixture alone was ~ 25% lower then when cell extract (no thrombin added) was present (not shown) which was essentially the same as the signal observed in the presence of cell extract and specific competitor. (C) Time course of prothrombin to thrombin conversion catalyzed by Factor Xa monitored by thrombin beacon. (D) Detection of thrombin in plasma. Data shown are averages and standard deviation from 4 independent measurements. The volumes of plasma used (per 20 μl assay mixture) were: 0.005 μl (light grey bars); 0.015 μl (dark grey bars); 0.045 μl (black bars). "Specific" refers to unlabeled thrombin aptamer competitor (THR7) whereas "nonspecific" refers to random sequence 30 nt DNA. Signal in panels A, B and D corresponds to a ratio of acceptor to donor emission measured with donor excitation. Signals were normalized to value of 1 for beacon mixture alone (panels A and D) and beacon mixture in the presence of cell extract (panel B). Panel C shows raw acceptor fluorescence intensity (with donor excitation).

[00042] Figure 26 depicts sensor variants.

[00043] Figure 27 depicts the experimental demonstration of sensor design shown in Fig. 26F. **(A)** Principle of sensor function. **(B)** Increase of sensitized acceptor fluorescence upon titration of increasing concentrations of DNA binding protein to the mixture of donor and acceptor labeled sensor components.

[00044] Figure 28 depicts the experimental demonstration of functioning of sensor design shown in Fig. 26G. **(A)** Principle of sensor function. **(B)** Increase of sensitized acceptor fluorescence (emission spectrum labeled with "+") upon addition of ss DNA containing two distinct sequence elements complementary to sensor elements to the mixture of two donor and acceptor labeled sensor components (spectrum labeled with "-").

[00045] Figure 29 depicts the experimental demonstration of the increased specificity of our sensor design compared to assays based on a single, target macromolecule-recognizing element.

[00046] Figure 30 depicts methods for preparing aptamers for the sensors illustrated in Fig. 26.

[00047] Figure 31 summarizes the selection of the aptamer binding to thrombin at an epitope distinct from the binding site of the G15D aptamer.

[00048] Figure 32 depicts the demonstration of the functional thrombin sensor comprising Texas Red-labeled THR27 and fluorescein-labeled THR35 or THR36, which contain sequence corresponding to that of clones 20-26 of Figure 31, panel C.

[00049] Figure 33 summarizes the simultaneous selection of two aptamers binding to the thrombin at two distinct epitopes.

[00050] Figure 34 summarizes the selection of the aptamer binding to CRP protein at a site distinct from the DNA binding site of that protein.

DETAILED DESCRIPTION OF THE INVENTION

[00051] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods or materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

[00052] The term "aptamer" refers to any polynucleotide, generally a RNA or a DNA that has a useful biological activity in terms of biochemical activity, molecular recognition or binding attributes. Usually, an aptamer has a molecular activity such as binding to a polypeptide at a

specific epitope (region) of the polypeptide or having an enzymatic activity. It is generally accepted that an aptamer, which is specific in its binding to any polypeptide, may be synthesized and/or identified by in vitro evolution methods.

[00053] The phrase "natural cognate binding element sequence" refers to a nucleotide sequence that serves as a binding site for a nucleic acid binding factor. Preferably the natural cognate binding element sequence is a naturally occurring sequence that is recognized by a naturally occurring nucleotide binding factor.

[00054] The term "molecular-recognition construct" refers to a construct that contains an "epitope binding agent" and can serve as a "molecular beacon". Preferably, a molecular-recognition construct also contains a "signaling oligo" and a "label". A first molecular-recognition construct and a second molecular-recognition construct may be joined together by a "linker" to form a "bivalent molecular-recognition construct." "Molecular beacon" refers to any chemical-based system for detecting or quantifying the presence of an analyte, polypeptide or other biomolecules, macromolecular complex comprising a biomolecules or a biological coregulator using a measurable read-out system as the detection method. An molecular-recognition construct or bivalent molecular-recognition construct is a particular type of molecular beacon with improved specificity and sensitivity.

[00055] The term "aptamer construct" refers to a construct that contains an aptamer or a natural cognate binding element sequence and can serve as a "molecular beacon". Preferably, an aptamer construct also contains a "signaling oligo" and a "label". A first aptamer construct and a second aptamer construct may be joined together by a "linker" to form a "bivalent aptamer construct." An aptamer construct is a subset of molecular-recognition construct. An aptamer construct or bivalent aptamer construct is also a particular type of molecular beacon with improved specificity and sensitivity.

[00056] The term "antibody" generally means a polypeptide or protein that recognizes and can bind to an epitope of an antigen. An antibody, as used herein, may be a complete antibody as understood in the art, i.e., consisting of two heavy chains and two light chains; or an antibody may be a fragment of a complete antibody, such as a Fab fragment or a peptide comprising a hypervariable region.

[00057] The term "epitope binding agent" refers to any substance that is capable of binding to a specific epitope of an antigen, a polypeptide, protein or macromolecular complex. Non-limiting examples of epitope binding agents include ligands and fragments of ligands, receptors and fragments of receptors, antibodies and fragments of antibodies, aptamers and other polynucleotides, coenzymes and other coregulators, and allosteric molecules and ions.

Preferred epitope binding agents include aptamers, natural cognate binding element sequences, antibodies and fragments thereof.

[00058] The term "epitope" refers generally to a particular region of an antigen, a hapten, a molecule, a polymer, a prion, a virion, a cell, a peptide, polypeptide, protein, or macromolecular complex. An epitope may consist of a small peptide derived from a larger polypeptide. An epitope may be a two or three dimensional surface or surface feature of a polypeptide, protein or macromolecular complex that comprises several non-contiguous peptide stretches or amino acid groups.

[00059] The term "signaling oligo" means a short (generally 2 to 15 nucleotides, preferably 5 to 7 nucleotides in length) single stranded polynucleotide. Preferably, a first signaling oligo sequence is complementary to the second signaling oligo. Preferably, the first signaling oligo and the second signaling oligo can not form a stable association with each other through hydrogen bonding unless the first and second signaling oligos are brought into close proximity to each other through the mediation of a third party agent.

[00060] As used herein, the term "linker" or "linker molecule" refers to any polymer attached to an aptamer or aptamer construct. The attachment may be covalent or non-covalent. It is envisioned that the linker can be a polymer of amino acids or nucleotides. A preferred linker molecule is flexible and does not interfere with the binding of a nucleic acid binding factor to the set of nucleic acid components. A preferred linker molecule is comprised of 12 moieties of the Spacer 18 phosphoramidate (Glen Research, Sterling, VA). Another preferred linker molecule is poly dT.

[00061] The phrase "in vitro evolution" generally means any method of selecting for an aptamer which binds to a biomolecule, particularly a peptide or polypeptide. In vitro evolution is also known as "in vitro selection", "SELEX" or "systematic evolution of ligands by exponential enrichment." Briefly, in vitro evolution involves screening a pool of random polynucleotides for a particular polynucleotide that binds to a biomolecule or has a particular activity that is selectable. Generally, the particular polynucleotide (i.e, aptamer) represents a very small fraction of the pool, therefore, a round of aptamer amplification, usually via polymerase chain reaction, is employed to increase the representation of potentially useful aptamers. Successive rounds of selection and amplification are employed to exponentially increase the abundance of the particular and useful aptamer. In vitro evolution is described in Famulok, M.; Szostak, J. W., In Vitro Selection of Specific Ligand Binding Nucleic Acids, *Angew. Chem.* 1992, *104*, 1001. (*Angew. Chem. Int. Ed. Engl.* 1992, *31*, 979-988.); Famulok, M.; Szostak, J. W., Selection of Functional RNA and DNA Molecules from Randomized Sequences, *Nucleic Acids and Molecular Biology*, Vol 7, F. Eckstein, D. M. J. Lilley, Eds.,

Springer Verlag, Berlin, 1993, pp. 271; Klug, S.; Famulok, M., All you wanted to know about SELEX; *Mol. Biol. Reports* 1994, *20*, 97-107; and Burgstaller, P.; Famulok, M. Synthetic ribozymes and the first deoxyribozyme; *Angew. Chem.* 1995, *107*, 1303-1306 (*Angew. Chem. Int. Ed. Engl.* 1995, *34*, 1189-1 192), which are incorporated herein by reference.

[00062] In the practice of this invention, in vitro evolution is used to generate aptamers that bind to distinct epitopes of any given polypeptide or macromolecular complex. Aptamers are selected against "substrates", which contain the epitope of interest. As used herein, a "substrate" is any molecular entity that contains an epitope to which an aptamer can bind and that is useful in the selection of an aptamer.

[00063] The term "label", as used herein, refers to any substance attachable to a polynucleotide, polypeptide, aptamer, nucleic acid component, or other substrate material, in which the substance is detectable by a detection method. Non-limiting examples of labels applicable to this invention include but are not limited to luminescent molecules, chemiluminescent molecules, fluorochromes, fluorescent quenching agents, colored molecules, radioisotopes, scintillants, massive labels (for detection via mass changes), biotin, avidin, streptavidin, protein A, protein G, antibodies or fragments thereof, Grb2, polyhistidine, Ni^{2+}, Flag tags, myc tags, heavy metals, enzymes, alkaline phosphatase, peroxidase, luciferase, electron donors/acceptors, acridinium esters, and colorimetric substrates. The skilled artisan would readily recognize other useful labels that are not mentioned above, which may be employed in the operation of the present invention.

[00064] As used herein, "detection method" means any method known in the art to detect a molecular interaction event. The phrase "detectable signal", as used herein, is essentially equivalent to "detection method." Detection methods include detecting changes in mass (e.g., plasmin resonance), changes in fluorescence (e.g., FRET, FCCS, fluorescence quenching or increasing fluorescence, fluorescence polarization), enzymatic activity (e.g., depletion of substrate or formation of a product, such as a detectable dye – NBT-BCIP system of alkaline phosphatase is an example), changes chemiluminescence or scintillation (e.g., scintillation proximity assay, luminescence resonance energy transfer, bioluminescence resonance energy transfer and the like).

[00065] As used herein, the term "analyte" refers generally to a ligand, chemical moiety, compound, ion, salt, metal, enzyme, secondary messenger of a cellular signal transduction pathway, drug, nanoparticle, environmental contaminant, toxin, fatty acid, steroid, hormone, carbohydrate, amino acid, peptide, polypeptide, protein or other amino acid polymer, microbe, virus or any other agent which is capable of binding to a polypeptide, protein or

macromolecular complex in such a way as to create an epitope or alter the availability of an epitope for binding to an aptamer.

[00066] As used herein, the term "macromolecular complex" refers to any composition of matter comprising a macromolecule. Preferably, these are complexes of one or more macromolecules, such as polypeptides, lipids, carbohydrates, nucleic acids, natural or artificial polymers and the like, in association with each other. The association may involve covalent or non-covalent interactions between components of the macromolecular complex. Macromolecular complexes may be relatively simple, such as a ligand bound polypeptide, relatively complex, such as a lipid raft, or very complex, such as a cell surface, virus, bacteria, spore and the like . Macromolecular complexes may be biological or non-biological in nature.

[00067] In one embodiment, the invention is directed to a method of detecting a polypeptide in a sample comprising the steps of contacting a sample with a first molecular-recognition construct and a second molecular-recognition construct, then detecting the stable interaction of the first and second molecular-recognition constructs via a detection method. Several useful molecular-recognition construct combination (sensor) variants are envisioned by the inventor, which are graphically depicted in figure 26. Panel A depicts a sensor variant comprising two aptamers recognizing two distinct epitopes of a protein. Panel B depicts a sensor variant comprising a double stranded polynucleotide containing binding site for DNA binding protein and an aptamer recognizing a distinct epitope of the protein. Panel C depicts a sensor variant comprising an antibody and an aptamer recognizing distinct epitopes of the protein. Panel D depicts a sensor variant comprising a double stranded polynucleotide containing a binding site for a DNA binding protein and an antibody recognizing a distinct epitope of the protein. Panel E depicts a sensor variant comprising two antibodies recognizing two distinct epitopes of the protein. Panel F depicts a sensor variant comprising two double stranded polynucleotide fragments recognizing two distinct sites of the protein. Panel G depicts a sensor variant comprising two single stranded polynucleotide elements recognizing two distinct sequence elements of another single stranded polynucleotide. Panel H depicts a sensor variant that allows for the direct detection of formation of a protein-polynucleotide complex using a double stranded polynucleotide fragment (containing the binding site of the protein) labeled with a first signaling oligonucleotide and the protein labeled with a second signaling oligonucleotide. Panel I depicts a sensor variant that allows for the direct detection of the formation of a protein-protein complex using two corresponding proteins labeled with signaling oligonucleotides.

[00068] In a preferred embodiment, the first and second molecular-recognition constructs are aptamer constructs, such that the first aptamer construct contains an aptamer or a naturally

occurring nucleic acid sequence that recognizes an epitope on a polypeptide (i.e., the first epitope) and the second aptamer construct contains an aptamer or a naturally occurring nucleic acid sequence that recognizes a separate epitope (i.e., the second epitope) on the same polypeptide (Figure 26, panels A and B). Preferably, the first aptamer construct and the second aptamer construct each contain a short single stranded oligonucleotide sequence (signaling oligo) such that the short single stranded oligonucleotide of the first aptamer construct (i.e., first signaling oligo) is complementary to the short single stranded oligonucleotide of the second aptamer construct (i.e., second signaling oligo). Without wishing to be bound by theory, the signaling oligos should be short enough so that they can not form a stable interaction with each other in the absence of the polypeptide, which is capable of bringing the two aptamer constructs together. Preferably, the signaling oligos are at least 5 nucleotides long, and no more that 7 nucleotides long.

[00069] Preferably, the first aptamer construct contains a first label and the second aptamer construct contains a second label, such that, in the presence of a polypeptide that contains the first epitope and the second epitope, the first and second labels interact to produce a detectable signal that signifies the presence or amount of polypeptide present in the sample. Preferably, the first label is a fluorescence donor and the second label is a fluorescence recipient and the detection method is a detection of a change in fluorescence signal output.

[00070] Optionally, the first aptamer construct may be fixed to a surface, the second aptamer construct may be fixed to a surface, or both may be fixed to a surface. (Surfaces can be microtitre plates, test tubes, beads, resins and other polymers and the like). In a preferred embodiment, the first aptamer construct and the second aptamer construct may be joined with each other by a flexible linker to form a bivalent aptamer. Preferred flexible linkers include Spacer 18 polymers and deoxythymidine ("dT") polymers.

[00071] In another embodiment, the first and second aptamers may be used to detect macromolecular complexes in a sample. In this embodiment, the first epitope is preferably on one polypeptide and the second epitope is on another polypeptide, such that when a macromolecular complex is formed, the one and another polypeptides are bought into proximity, resulting in the stable interaction of the first aptamer construct and the second aptamer construct to produce a detectable signal, as described above. Also, the first and second aptamer constructs may be fixed to a surface or to each other via a flexible linker, as described above.

[00072] In another embodiment, the first and second aptamers may be used to detect analytes in a sample. In this embodiment, when the analyte is bound to a polypeptide or macromolecular complex, a first or second epitope is created or made available to bind to a first or second

aptamer construct. Thus, when a an analyte is present in a sample that contains its cognate polypeptide or macromolecular binding partner, the first aptamer construct and the second aptamer construct are brought into stable proximity to produce a detectable signal, as described above. Also, the first and second aptamer constructs may be fixed to a surface or to each other via a flexible linker, as described above.

[00073] In another embodiment, the invention is directed to a method of making a set of aptamer constructs, comprising a first and second aptamer construct, comprising the steps of (a) selecting a first aptamer against a first substrate, which comprises a first epitope, and selecting a second aptamer against a second substrate, which comprises a second epitope, wherein the first aptamer is capable of binding to the first epitope and the second aptamer is capable of binding to the second epitope, (b) attaching a first label to the first aptamer and attaching a second label to the second aptamer, (c) attaching a first signaling oligo to the first aptamer and attaching a second signaling oligo to the second aptamer, wherein the second signaling oligo is complementary to the first signaling oligo, and (d) such that (i) the first aptamer construct comprises the first aptamer, the first label and the first signaling oligo, and (ii) the second aptamer construct comprises the second aptamer, the second label and the second signaling oligo. Preferably, the aptamers are selected using in vitro evolution methods (supra), however, natural DNA binding elements may be used in the practice of this invention.

[00074] In a preferred embodiment, the first substrate is a polypeptide and the second substrate is the polypeptide bound to the first aptamer, wherein the first aptamer masks the first epitope, such that the first epitope is not available for the second aptamer to bind. Alternatively, the first aptamer may be replaced by a first aptamer construct that contains (i) the first aptamer and signaling oligo, or (ii) the first aptamer, signaling oligo and label, thereby producing a second substrate that allows for the selection of the optimum second aptamer or aptamer construct for signal detection. As a further step, the first and second aptamer constructs may then be joined together by a flexible linker, as described above.

[00075] In an alternate preferred embodiment, the first substrate is a peptide consisting essentially of the first epitope and the second substrate is a peptide consisting essentially of the second epitope. Thus, in this alternate embodiment, there is no need to mask an epitope in the production or selection of aptamers. Again, the first and second aptamer constructs created by this method may be linked together by a flexible linker, as described above.

[00076] In another embodiment, the invention is directed to a bivalent aptamer construct comprising a first aptamer, a first label, a first signaling oligo, a second aptamer, a second label, a second signaling oligo and a linker, wherein the first aptamer is capable of binding to

a first epitope and the second aptamer is capable of binding to a second epitope. The first and second epitopes may be on the same polypeptide, on different polypeptides of a macromolecular complex, or present on a polypeptide that is bound to an analyte. The flexible linker is preferably a polymer that does not interfere with the function of the aptamers. Prefered flexible linkers include deoxythymidine polymer (poly dT) and Spacer 18 polymer. However, the skilled artisan in the practice of this invention may substitute any number of linkers.

[00077] Alternatively, the bivalent aptamer construct may not have labels for detection. It is envisioned that these alternative bivalent aptamer constructs may be used much like antibodies to detect molecules, bind molecules, purify molecules (as in a column or pull-down type of procedure), block molecular interactions, facilitate or stabilize molecular interactions, or confer passive immunity to an organism. It is further envisioned that the bivalent aptamer construct can be used for therapeutic purposes. This invention is truly powerful in that it enables the skilled artisan to build any combination of aptamers that recognize any two or more disparate epitopes form any number of molecules into a bivalent, trivalent, or other multivalent aptamer construct to pull together those disparate molecules to test the effect or to produce a desired therapeutic outcome. For example, a bivalent aptamer construct may be constructed to facilitate the binding of a ligand to its receptor in a situation wherein the natural binding kinetics of that ligand to the receptor is not favorable (e.g., insulin to insulin receptor in patients suffering diabetes.)

[00078] In another embodiment, the invention is directed to a kit comprising a first epitope binding agent, to which is attached a first label, and a second epitope binding agent, to which is attached a second label, wherein (a) when the first epitope binding agent and the second epitope binding agent label bind to a first epitope of a polypeptide and a second epitope of the polypeptide, respectively, (b) the first label and the second label interact to produce a detectable signal. In a preferred embodiment the epitope binding agent is an aptamer construct, which comprises an aptamer, a label and a signaling oligo. However, the epitope binding agent may be an antibody or antibody fragment. The kit is useful in the detection of polypeptides, analytes or macromolecular complexes, and as such, may be used in research or medical/veterinary diagnostics applications.

[00079] In yet another embodiment, the invention is directed to a method of diagnosing a disease comprising the steps of (a) obtaining a sample from a patient, (b) contacting the sample with a first epitope binding agent and a second epitope binding agent, and (c) detecting the presence of a polypeptide, analyte or macromolecular complex in the sample using a detection method, wherein the presence of the polypeptide, analyte or macromolecular complex in the sample indicates whether a disease is present in the patient. In a preferred embodiment, (a) the first

epitope binding agent is a first aptamer to which a first label and a first signaling oligo are attached, (b) the second epitope binding agent is a second aptamer to which a second label and a second signaling oligo, which is complementary to the first signaling oligo, are attached, and (c) the detection method is a fluorescence detection method, wherein, (d) when the first aptamer binds to the polypeptide and the second aptamer binds to the polypeptide, (e) the first signaling oligo and the second signaling oligo associate with each other, and (f) the first label is brought into proximity to the second label such that a change in fluorescence occurs. Prefered samples include blood, urine, ascites, cells and tissue samples/biopsies. Prefered patients include humans, farm animals and companion animals.

[00080] In yet another embodiment, the invention is directed to a method of screening a sample for useful reagents comprising the steps of (a) contacting a sample with a first epitope binding agent and a second epitope binding agent, and (b) detecting the presence of a useful reagent in the sample using a detection method. Preferred reagents include a polypeptide, which comprises a first epitope and a second epitope, an analyte that binds to a polypeptide (in which case the method further comprises the step of adding the polypeptide to the screening mixture) and a potential therapeutic composition. In a preferred embodiment, (a) the first epitope binding agent is a first aptamer to which a first label and a first signaling oligo are attached, (b) the second epitope binding agent is a second aptamer to which a second label and a second signaling oligo, which is complementary to the first signaling oligo, are attached, and (c) the detection method is a fluorescence detection method, wherein, (d) when the first aptamer binds to the polypeptide and the second aptamer binds to the polypeptide, (e) the first signaling oligo and the second signaling oligo associate with each other, and (f) the first label is brought into proximity to the second label such that a change in fluorescence occurs.

[00081] Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims, which follow the examples.

EXAMPLE 1: GENERAL METHOD FOR PREPARING SPECIFIC APTAMER CONSTRUCTS

Introduction

[00082] Disclosed is a method for the rapid and sensitive detection in a sample of proteins, protein complexes, or analytes that bind to proteins. This method is based on the protein-driven

association of two nucleic acid constructs containing aptamers that recognize two distinct epitopes of a protein (a.k.a. "aptamer constructs") (Fig. 1A). These two aptamer constructs also contain short complementary signaling oliginucleotides attached to the aptamers through a flexible linker. Upon the simultaneous binding of the two aptamers to the target protein, the complementary oligonucleotides (a.k.a. "signaling oligos") are brought into relative proximity which promotes their association to form a stable duplex. Attaching fluorescence probes to the ends of the signaling oligos provides a means of detecting the protein-induced association of the two aptamer contructs (Fig. 1A). In the case of proteins which possess natural nucleic acid binding activity, one of the aptamers can be substituted with a nucleic acid sequence containing the natural binding site of the protein (Fig. 1B).

[00083] Development or selection of aptamers directed to two distinct epitopes of a given protein is an essential step in developing the aptamer constructs depicted in Fig. 1. Review of the available literature on aptamers reveals at least two possible approaches to achieve this goal. The first approach is to perform in vitro selection (a.k.a. in vitro evolution) of nucleic acid aptamers using different methods for the separation of protein-bound and protein-unbound nucleic acid aptamers. The rationale here is that in these different partitioning methods different regions of the protein is preferentially displayed resulting in aptamers directed to different regions of the protein surface. Aptamers selected to thrombin (infra) are an example of such an approach.

[00084] The in vitro selection of a first aptamer using as a substrate thrombin immobilized on agarose beads resulted in aptamer binding the thrombin at the heparin exosite. Additional in vitro selection using as a substrate the thrombin-first aptamer complex, which was bound to nitrocellulose as the partitioning method, resulted in second aptamer binding the thrombin at the fibrinogen exosite.

[00085] While useful, this partitioning approach relies on the chance selection of distinct epitopes rather than on intelligent design. The second approach is to raise or select the aptamers using as substrates peptides that correspond to selected regions of the target protein molecule. There is evidence in the art which demonstrates that such strategy can be used to develop aptamers capable of recognizing the intact protein from which the peptide used as a substrate for aptamer development was derived. Furthermore, this approach has been widely used to generate antibodies which recognize an intact protein.

[00086] The general approach for preparing a set of aptamers directed to an epitope of the protein distinct from the binding site of the first aptamer can be also used for proteins that possess natural DNA binding activity. That is, co-aptamers, which bind the substrate protein at a site

distinct from the natural DNA binding site, can be produced. Co-aptamers produced by this method are optimized for functioning in the molecular detection method depicted in Fig.1.

Results and Discussion

[00087] Fig. 2 illustrates the overall design of the methods described above. Selection of the co-aptamer is performed using substrate protein pre-bound to the first aptamer (Fig. 2A). Alternatively, selection of the co-aptamer is performed using protein pre-bound to it's natural nucleic acid binding site (Fig. 2B). A short (5-7 nt) single-stranded oligonucleotide, i.e., the signaling oligo (Fig. 2), is attached to the first aptamer by a flexible linker. Random DNA (or RNA) to be used for selection of co-aptamers is flanked by uniform sequences for the purpose of PCR amplification. One of these uniform flanking sequences contains at its end a sequence that is complementary to the signaling oligo of the first aptamer, i.e., the other signaling oligo (Fig. 2). Thus, the creation and selection of co-aptamers using such a random DNA (or RNA) construct is biased towards aptamers that are able to bind to the substrate protein at a site distinct from the epitope of the first aptamer, and are able to form a duplex between the signaling oligo of the first aptamer. The degree of the bias in the selection is adjusted by varying the length of the signaling oligo of the first aptamer and complementary signaling oligo of the second aptamer.

EXAMPLE 2: METHODS AND APTAMERS FOR DETECTING THROMBIN

Introduction

[00088] The inventors of the instant invention have developed a methodology for detecting DNA binding proteins, as described in Heyduk, T. and Heyduk, E. Molecular beacons for detecting DNA binding proteins. *Nature Biotechnology*, **20**, 171-176, 2002, Heyduk, E., Knoll, E., and Heyduk, T. Molecular beacons for detecting DNA binding proteins: mechanism of action, *Analyt. Biochem.* **316**, 1-10, 2003, and copending patent applications number 09/928,385, which issued as U.S. Pat. No. 6,544,746, 10/062,064, PCT/US02/24822 and PCT/US03/02157, all of which are incorporated herein by reference. This methodology is based on splitting the DNA binding site for a protein into two DNA "half-sites" (Fig. 3A). Each of the resulting "half-sites" contains a short complementary single-stranded region of the length designed to introduce some propensity for the two DNA "half-sites" to associate recreating the duplex containing the fully functional cognate protein binding site. This propensity is designed to be low such that in the absence of the protein only a small fraction of DNA half-sites will associate. When the protein is present in the reaction mixture, it will bind only to the duplex containing a full and functional binding site. This selective binding

drives the association of DNA half-sites and this protein-dependent association can be used to generate a spectroscopic or other signal reporting the presence of the target protein.

[00089] The term "molecular beacons" is used in the scientific literature to describe this assay in order to emphasize the fact that the selective recognition and generation of the reporting signal occur simultaneously. Molecular beacons for DNA binding proteins have been developed for several proteins (Heyduk and Heyduk, 2002) illustrating their general applicability. Their physical mechanism of action has been established (Heyduk, Knoll and Heyduk, 2003) and they have been also used as a platform for the assay detecting the presence of ligands binding to DNA binding proteins (Heyduk, E., Fei, Y., and Heyduk, T. Homogenous fluorescence assay for cAMP. *Combinatorial Chemistry and High-throughput Screening* **6**, 183-194, 2003). While already very useful, this assay is limited to proteins which exhibit natural DNA binding activity.

[00090] It has been well established that nucleic acid (DNA or RNA) aptamers capable of specific binding to proteins lacking natural DNA binding activity can be produced by in vitro selection methods (Ellington, A.D., and Szostak, J.W. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818-822, 1990; Tuerk, C., and Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505-510, 1990; Gold, L., Polisky, B., Uhlenbeck, O. & Yarus, M. Diversity of Oligonucleotide Function. *Ann. Rev. Biochem.* **64**, 763-797, 1995; and Wilson, D.S. & Szostak, J.W. In vitro selection of functional nucleic acids. *Ann. Rev. Biochem.* **68**, 611-647, 1999; all of which are incorporated herein by reference). In vitro selection involves selection of nucleic acid sequences, which bind to a specific substrate target, from a pool of random DNA sequences by cycles of binding, washing out unbound sequences and PCR amplification of target-bound sequences. Numerous examples of the successful selection of aptamers that specifically bind to a variety of proteins as well as other target molecules (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Gold et alia, 1995; Wilson and Szostak, 1999) provide a strong indication that producing aptamers to any and all proteins is possible.

[00091] Described in this example is the novel concept of nucleic acid-based molecular beacons for protein detection, which is not limited to proteins with natural DNA binding activity. The example of thrombin (infra) provides experimental validation for this invention.

Results and Discussion

[00092] Fig. 3B illustrates the overall concept of molecular beacons recognizing any target protein. This design shares some general similarities with molecular beacons for DNA binding proteins described previously and supra (Fig. 3A). Instead of splitting the DNA duplex containing the natural binding site for a protein into the two "half-sites", two aptamers

recognizing two different epitopes of the protein are used as functional equivalents of the "half-sites". Short complementary oligonucleotides (signaling oligos) containing the fluorophore (first label) and the quencher (second label) are attached to the two aptamers via a flexible linker (Fig. 3B). In the absence of the target protein, the two aptamer constructs do not associate since the complementary signal oligos are too short to promote association. In the presence of the target protein, the preferential binding of the protein to the two aptamers should drive the association of the two aptamer constructs resulting in a fluorescence signal change due to bringing the first and second labels into a close proximity.

[00093] Thrombin was selected as a model non-DNA-binding-protein system to provide experimental verification of the concept illustrated in Fig. 3B. Two laboratories have previously identified DNA aptamers which selectively recognized two distinct epitopes of the protein (Bock, L.C., Griffin, L.C., Latham, J.A., Vermass, E.H., and Toole, J.J. Selection of single-stranded DNA molecules that bind and inhibit human thrombin, *Nature* **355,** 564-566, 1992; and Tasset, D.M., Kubik, M.F., and Steiner, W. Oligonucleotide inhibitors of human thrombin that bind distinct epitopes, *J. Mol. Biol.* **272,** 688-98, 1997, which are incorporated herein by reference). One aptamer (G15D; THR4 in Fig. 4) was shown to bind to the heparin exosite whereas the other aptamer (60-18 [29]; THR3 in Fig. 4) was shown to bind to the fibrinogen exosite. As a first step towards developing a set of aptamer constructs useful for recognizing thrombin, we prepared various aptamer constructs in which the above aptamers were covalently linked by flexible linkers (Fig. 4). The primary purpose of these experiments was to determine if indeed linking the two aptamer constructs with a flexible linker would produce a bivalent aptamer construct capable of binding thrombin with higher affinity compared to a set of individual aptamer constructs. A second purpose of these experiments was to establish a suitable length of the linker and the appropriate orientation of 5' and 3' ends of the two aptamers with respect to the linker.

[00094] Individual aptamers were labeled with fluorescein (THR1 and THR2 in Fig. 4) to facilitate determination of the affinity of various constructs for thrombin. Formation of a complex between thrombin and fluorescein-labeled 60-18 [29] aptamer (THR1) could be conveniently followed by fluorescence polarization (Fig. 5A) whereas binding of the fluorescein-labeled G15D aptamer (THR2) could be followed by changes in fluorescence intensity (Fig. 5B). Both aptamers bound thrombin in the nanomolar concentration range (Fig. 5A and 5B). Quantitative analysis of the binding in the case of THR2 (Fig. 5C) returned the value of K_d of 6.3 nM. This is somewhat of a higher affinity than that previously suggested (Bock et alia, 1992), which could be explained by the true equilibrium binding assay used by us vs. the non-equilibrium methodology used previously. When the binding of THR2 was performed in the presence of 10-fold excess of unlabeled 60-18 [29] aptamer (THR3) (Fig.

5D) only a small and insignificant decrease in affinity was observed. This shows that indeed G15D and 60-18 [29] aptamers bind independently to two distinct epitopes of thrombin.

[00095] In the next step the ability of various aptamer constructs illustrated in Fig. 4 to compete with THR2 for binding to thrombin was evaluated. Fig. 6 illustrates the manner in which these experiments were performed. Fluorescence spectra of HR2 were recorded in the presence and absence of thrombin (Fig. 6A). Thrombin produced ~50% increase in fluorescence of THR2. Unlabeled competitor aptamer constructs were then added (Figs. 6 B-D). A small effect of thrombin on the fluorescence of THR2 in the presence of a competitor would be a hallmark of an efficient competitor. THR3 was not a competitor (Fig. 6B) in agreement with the data shown in Fig. 5 C and D. THR4 (an unlabeled variant of THR2) was able to compete as expected (Fig. 6C). However, THR7 (one of the bivalent aptamer constructs) was a much better competitor than THR4 (Fig. 6D). No fluorescence change of THR2 in the presence of thrombin was detected when THR7 was present in solution. Fig. 7 shows a summary of the competition experiments with all of the constructs shown in Fig. 4.

[00096] All bivalent aptamer constructs were shown to bind to thrombin much tighter (K_d's in pM range) than individual aptamers, thus providing validation of the expectation that linking two aptamers, which recognize two different epitopes of the protein, with flexible linkers should produce high-affinity thrombin ligands. Additionally, these data showed that linking two aptamers by a longer linker containing 10 Spacer18 units produced slightly better affinity for thrombin (compare binding of THR5 vs. THR6). Also, these data showed that orientation of the aptamers with respect to the linker as in THR7 produced better affinity (compare affinity of THR6 vs. THR7). Thus, in all subsequent experiments constructs having an aptamer orientation as in THR7 were used.

[00097] The purpose of experiments shown in Fig. 8 was to demonstrate that both epitopes of thrombin are important for high affinity binding of bivalent aptamer constructs. Direct competition between binding of THR2 and the bivalent aptamer construct provided evidence that the epitope recognized by THR2 (heparin exosite) was necessary for bivalent aptamer binding. To demonstrate that the second epitope was also important, we compared the ability of a bivalent aptamer construct (THR5) to compete with THR2 for binding to thrombin in the absence and presence of excess of unlabeled THR3. We expected that if THR5 needs both thrombin epitopes for high-affinity binding, in the presence of THR3 one should observe diminished ability of THR5 to compete with THR2. This is exactly what has been observed in experiments illustrated in Fig. 8. THR5 alone was a very effective competitor for THR2 (compare Fig. 8D with 8A). THR3 alone was not a competitor for THR2 (compare Fig. 8A and C). THR5 in the presence of THR3 was a worse competitor than THR5 alone (compare

Fig. 8B with 8C). We therefore concluded that high-affinity binding of the bivalent aptamer constructs to thrombin involves both first and second aptamer epitopes.

[00098] The bivalent aptamer construct-thrombin complex was stable enough to survive electrophoresis in native polyacrylamide gel (Fig. 9A). We took advantage of this attribute to determine the stoichiometry of the complex using EMSA to follow THR7-thrombin complex formation. We performed a titration of THR7 with thrombin at high concentrations of both molecules. Under these conditions, the binding should be stoichiometric. The plot of the complex formed vs. the ratio of thrombin to THR7 did in fact show a 1:1 stoichiometry of the complex (Fig. 9B).

[00099] The experiments illustrated in Fig. 10 and 11 were performed to test if an alternative design of bivalent aptamer constructs could be used to prepare these constructs. We designed bivalent aptamer constructs shown in Fig. 10 such that they were made entirely of DNA, avoiding the use of non-DNA linker (poly dT was used as the linker in this case) (Fig. 10). This could potentially offer more flexibility in designing such constructs and could also lower the cost of making the aptamer constructs. Two aptamers were joined together by a DNA duplex at the end of flexible linkers (Fig. 10). This aspect of the invention was intended to mimic the design of signaling "beacons" (Fig. 3B) in which the signaling function involves formation of a DNA duplex at the end of the linkers connecting the aptamers to the duplex. Three different lengths of the poly dT linker were tested (7, 17 and 27 nt) to determine the minimal linker length requirement for high-affinity binding. Fig. 11 shows the results of simultaneous titration of the constructs shown in Fig. 10 with thrombin. Formation of aptamer construct-thrombin complexes was followed by EMSA. Each of the constructs bound thrombin with high affinity. However, it is clear that the construct with 7 nt poly dT linker had significantly lower affinity to thrombin compared to constructs with 17 and 27 nt linkers. This is best illustrated by inspecting a lane marked with the asterisk which shows that at this particular concentration of thrombin almost all of 17 and 27 nt poly dT linker constructs were bound by thrombin whereas a significant (~50%) fraction of the 7 nt poly dT construct remained unbound. In summary, the results described in Figs. 10 and 11 show that the alternative design of bivalent aptamer constructs illustrated in Fig. 10 is feasible and that at least 17 nt long poly dT linker connecting the aptamers with the DNA duplex is more optimal for binding of the constructs to thrombin.

[000100] The experimental data presented in Figs. 3-11 provided evidence that all necessary conditions for the signaling beacon shown in Fig. 3B to function were met in the case of thrombin and the two aptamers binding to two distinct region of thrombin. Based on the information provided by the experiments illustrated in Figs. 3-11, we designed and tested a thrombin signaling beacon. The beacon shown in Fig. 12A and B is a derivative of

THR16/THR17 bivalent aptamer construct. Aptamers were connected using 17 nt long poly dT linker to 7 nt complementary oligonucleotides (signaling oligos) labeled at 5' and 3' with fluorescein and dabcyl, respectively. Addition of thrombin to a mixture of THR8 and THR9 resulted in protein-dependent quenching of fluorescence intensity. No fluorescence change was observed upon addition of thrombin to THR9 in the absence of dabcyl-labeled partner (THR8). Clearly, these data show that indeed the expected thrombin-driven association between THR8 and THR9 (as illustrated in Fig. 12B) was observed and a functional thrombin signaling beacon was thus obtained.

[000101] The magnitude of the fluorescence change induced by thrombin, while very reproducible and specific, initially was not very large (~ 20%). We therefore sought to improve this property of the thrombin signaling beacon by replacing the poly dT linkers with the more flexible Spacer 18 linker (Fig. 13A&B). We reasoned that poly dT linkers, while flexible, exhibit some residual rigidity (Mills, J.B., Vacano, E., and Hagerman, P.J. Flexibility of single-stranded DNA: use of gapped duplex helices to determine the persistence lengths of poly(dT) and poly(dA), *J. Mol. Biol.* **285**, 245-57, 1999; which is incorporated herein by reference), which could impede the association of the signaling duplex when the two aptamers are bound to thrombin. The beacon shown in Fig. 13 differs only in the nature of the linkers from the beacon shown in Fig. 12. The remaining sequence is otherwise identical. Fig. 13 C shows that upon addition of thrombin to a mixture of THR20 and THR21, protein concentration-dependent quenching of fluorescence was observed whereas no change of fluorescence was detected when thrombin was added to THR21 alone. Response of the beacon to thrombin in the case of this particular beacon was much larger (a ~ 2-fold decrease in fluorescence). The degree of fluorescence signal change in this case was comparable to what we had previously observed with beacons for detecting DNA binding proteins (supra). We concluded thus that a functional thrombin beacon was obtained and that the design utilizing a more flexible Spacer18 linker resulted in a better signal change upon thrombin binding compared to the design with poly dT linker. We next conducted a series of experiments to further characterize the behavior of this thrombin beacon.

[000102] The experiment illustrated in Fig. 14 was conducted to provide confirmation that indeed the fluorescein-labeled aptamer construct (THR21) was incorporated into a stable complex in the presence of THR20 and thrombin. Fig. 15 shows the results, which illustrates the sensitivity of thrombin detection (Fig. 15A) and specificity of thrombin detection (Fig. 15B). Because the binding of thrombin to bivalent aptamer constructs was extremely tight (pM K_d's), and since the assay appears to be limited only by the sensitivity of detection of fluorescein signal, the sensitivity of thrombin detection could be manipulated by changing the concentration of the aptamer constructs. This is illustrated in Fig. 15 A where using 50 nM

THR21 and 75 nM THR20, ~ 10 nM of thrombin could be detected whereas, when 10 fold smaller concentrations of aptamer constructs were used (5 nM THR21 and 7.5 nM THR20), a 10 fold lower (~ 1nM) concentration of thrombin could be detected. Using even lower aptamer contruct concentrations (500 pM THR21 and 750 pM THR22), ~ 100 pM thrombin could be detected (not shown), but this low concentration of fluorescein-labeled aptamer construct is close to the limits of sensitivity of our instrumentation and the quality of the data was concomitantly decreased. To demonstrate the specificity of thrombin detection, we compared the response of the aptamer constructs to thrombin with the response to trypsin, a protease belonging to the same family as thrombin and sharing structural homologies with thrombin. No signal was detected upon addition of trypsin (Fig. 15B), indicating a high specificity of the aptamer constructs for thrombin.

[000103] Fig. 16 shows the results of competition experiments, in which the ability of various aptamer constructs to dissociate the preformed thrombin-aptamer construct complex was tested. The data obtained showed that all bivalent aptamer constructs were by far much more efficient competitors than any of the individual epitope-specific aptamers, in agreement with similar experiments performed with fluorescein-labeled individual aptamer (supra, THR2; Fig. 6). Among the bivalent aptamer constructs, THR18/THR19 (a construct with 27 nt long poly dT linker) and THR16/THR17 (a construct with 17 nt long poly dT linker) were the most efficient competitors followed by THR14/THR15 (a construct with 7 nt poly dT linker) and THR7 (which has a Spacer18 linker). It appears thus that although additional flexibility of Spacer18 linkers was beneficial in terms of the magnitude of fluorescence signal change produced by the aptamer construct signal change, it also resulted in somewhat reduced affinity for binding thrombin in comparison with the constructs containing more rigid poly dT linkers.

Conclusions

[000104] We obtained data provided basic physicochemical characterization of the bivalent aptamer constructs containing two aptamers recognizing two different epitopes of thrombin. The bivalent constructs exhibited much higher affinity for thrombin than the individual aptamers-components of the bivalent construct. This suggested that addition of thrombin to a mixture of aptamers "half-sites" should induce association of the two "half-sites" generating fluorescence signal as a result of bringing the fluorophore and the quencher to close proximity. Experiments with beacon constructs fully validated this prediction. We expect that it will be possible to develop analogous beacons for a large number of target proteins. We also note that the beacon design described here can be also adopted to improve beacons for detecting proteins exhibiting natural DNA binding activity (Fig. 1 A). In this case one of the

aptamers "half-sites" can be replaced with the DNA duplex (containing the protein binding site sequence) connected to signaling complementary oligonucleotide via flexible linker.

EXAMPLE 3: ANALYTE DETECTION IN A SAMPLE

Materials

[000105] Purified thrombin was a gift from Dr. Ray Rezaie (St. Louis University). Factor Xa, prothrombin, ovalbumin, bovine serum albumin, SSB, trypsin and plasma were purchased from Sigma (St. Louis, MO). HeLa cellular extracts were from ProteinOne (College Park, MD). Texas Red-NHS and Sybr Green were from Molecular Probes (Eugene, OR), Cy5-NHS and Cy3-NHS were from Amersham Biosciences (Piscataway, NJ), and AMCA-sulfoNHS was from Pierce (Rockford, IL). All other reagents were commercially available analytical grade.

[000106] Oligonucleotide constructs used throughout this work are listed in Table 1. Oligonucleotides were obtained from Keck Oligonucleotide Synthesis Facility at Yale University or from IDT (Coralville, IA). 5' fluorescein and 3' dabcyl were incorporated using appropriate phosphoramidates during oligonucleoide synthesis. All other fluorophores were incorporated into oligonucleotides by post-synthetic modification of oligonucleotides containing 5' amino or C6 amino-dT at appriopriate positions with NHS esters of the dyes. Oligonucleotides labeled with fluorescence probes were purified by reverse-phase HPLC as described previously (Heyduk, E.; Heyduk, T. *Anal. Biochem.* **1997**, 248, 216-227). Modification of oligonucleotides with europium chelate ((Eu^{3+})DTPA-AMCA) was performed by a two-step procedure described in Heyduk, E.; Heyduk, T.; Claus, P.; Wisniewski, J.R. *J. Biol. Chem.* **1997**, 272, 19763-19770. Concentrations of all oligonucleotides were calculated from UV absorbance at 260 nm after correction for the contribution of the fluorophore absorbance at 260 nm.

Table 1

Construct	***Sequence***	***Sequence Identifier***	***Description***
THR1	5' Fluorescein AGT CCG TGG TAG GGC AGG TTG GGG TGA CT	SEQ ID NO:1	60-18 [29][a] aptamer labeled with fluorescein
THR2	5' Fluorescein GGT TGG TGT GGT TGG	SEQ ID NO:2	G15D[b] aptamer labeled with fluorescein
THR3	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT	SEQ ID NO:3	60-18 [29] aptamer

THR4	GGT TGG TGT GGT TGG	SEQ ID NO:4	G15D aptamer
THR5	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT $(Spacer18)_5$ GG TTG GTG TGG TTG G	SEQ ID NO:5	60-18 [29] aptamer connected to G15D aptamer via 5 Spacer18 linkers
THR6	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT $(Spacer18)_{10}$ GGT TGG TGT GGT TGG	SEQ ID NO:6	60-18 [29] aptamer connected to G15D aptamer via 10 Spacer18 linkers
THR7	GGT TGG TGT GGT TGG $(Spacer18)_{10}$ AG TCC GTG GTA GGG CAG GTT GGG GTG ACT	SEQ ID NO:7	G15D aptamer connected to 60-18 [29] aptamer via 10 Spacer18 linkers
THR20	GGT TGG TGT GGT TGG $(Spacer18)_5$ C GCA TCT 3'dabcyl	SEQ ID NO:16	G15D aptamer connected via 5 Spacer18 linkers to 7 nt "signaling" oligonucleotide labeled with dabcyl at 3' end
THR21	5' fluorescein AGA TGC G $(Spacer18)_5$ AGT CCG TGG TAG GGC AGG TTG GGG TGA CT	SEQ ID NO:17	7 nt "signaling" oligonucleotide labeled at 5' with fluorescein connected to 60-18 [29] aptamer via 5 Spacer18 linkers
THR27	GGT TGG TGT GGT TGG $(Spacer18)_5$ (C6 amino-dT) C GCA TCT	SEQ ID NO:18	G15D aptamer connected via 5 Spacer18 linkers to 7 nt "signaling" oligonucleotide containing amino-dT (near its 5' end)
THR28	5' amino AGA TGC G $(Spacer18)_5$ AGT CCG TGG TAG GGC AGG TTG GGG TGA CT	SEQ ID NO:19	7 nt "signaling" oligonucleotide containing 5' amino connected to 60-18 [29] aptamer via 5 Spacer18 linkers
THR11	CTG TCG TTA GTG AAG GTT NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN AAC GCC ATA TCA CAG ACG	SEQ ID NO:20	Construct containing 33 nt random DNA sequence for thrombin aptamer selection
THR12	5' fluorescein CTG TCG TTA GTG AAG GTT	SEQ ID NO:21	Primer1 for THR11
THR13	5' biotin CGT CTG TGA TAT GGC GTT	SEQ ID NO:22	Primer2 for THR11

THR22	GGT TGG TGT GGT TGG (Spacer18)$_2$ GA CAG	SEQ ID NO:23	Co-aptamer for thrombin aptamer selection
THR25	GGT TGG TGT GGT TGG (Spacer18)$_5$ AC GA CAG	SEQ ID NO:24	Co-aptamer for thrombin aptamer selection
THR29	GAACGAGAGTGC XXXXX amino C GCA TCT	SEQ ID NO:25	ss DNA sensor component
THR32	5' fluorescein AGA TGC G XXXXX TTGAACTGGACC	SEQ ID NO:26	ss DNA sensor component
THR33	GGTCCAGTTCAA TT GCACTCTCGTTC	SEQ ID NO:27	target ss DNA for ss DNA sensor
THR42	GGT TGG TGT GGT TGG XX XXX AAC GAC AG	SEQ ID NO:28	co-aptamer for thrombin aptamer selection
THR43	CTG TCG TT (Spacer18)$_5$ TTGAGTCAGCGTCGAG CA NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN TTC ACT GTG CTG CGG CTA	SEQ ID NO:29	Construct containing 33 nt random DNA sequence for thrombin aptamer selection
THR44	5' fluorescein CTG TCG TT (Spacer18)$_5$ TTG AGT CAG CGT CGA GCA	SEQ ID NO:30	Primer1 for THR43
THR45	5'biotin TAGCCGCAGCACAGTG AA	SEQ ID NO:31	Primer2 for THR43
THR49	CACCTGATCGCTCCTC GT NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN CAG GAT GCA CAG GCA CAA	SEQ ID NO:32	Construct containing 30 nt random DNA sequence for simultaneous selection of two thrombin aptamers
THR50	AGCCGCCATTCCATAG TG NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN CAG GAT GCC GAT CAG GTG	SEQ ID NO:33	Construct containing 30 nt random DNA sequence for simultaneous selection of two thrombin aptamers
THR51	5' fluorescein CAC CTG ATC GCT CCT CGT	SEQ ID NO:34	Primer1 for THR49
THR52	5'biotin TTG TGC CTG TGC ATC CTG	SEQ ID NO:35	Primer2 for THR49
THR53	5' fluorescein-AGC CGC CAT TCC ATA GTG	SEQ ID NO:36	Primer3 for THR50
THR54	5'biotin CAC CTG ATC GGC ATC CTG	SEQ ID NO:37	Primer4 for THR50

THR35	5' fluorescein AGA TGC G (Spacer18)$_5$ AG GTT GGG GGT ACT AGG TAT CAA TGG GTA GGG TGG TGT AAC GC	SEQ ID NO:38	Thrombin sensor component
THR36	5' fluorescein AGA TGC G (Spacer18)$_5$ A GTG AAG GTT GGG GGT ACT AGG TAT CAA TGG GTA GGG TGG TGT AAC GCC ATA T	SEQ ID NO:39	Thrombin sensor component
MIS10X3	AAC GCA ATA AAT GTG AAG TAG ATC ACA TTT TAG GCA CC (Spacer18)$_5$ GA TGGCT	SEQ ID NO:40	co-aptamer for CRP aptamer selection
MIS12	AGCCA T CTA ACT ATT CCC NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN GAG CGA GAA ATT CTA GGT	SEQ ID NO:41	Construct containing 33 nt random DNA sequence for CRP aptamer selection
MIS11	GGT GCC TAA AAT GTG ATC TAC TTC ACA TTT ATT GCG TT	SEQ ID NO:42	Complement to MIS10X3
MIS13	5'-fluorescein - AGC CA T CTA ACT ATT CCC	SEQ ID NO:43	Primer1 for MIS10X3
MIS14	5' biotin- ACC TAG AAT TTC TCG CTC	SEQ ID NO:44	Primer2 for MIS10X3

Fluorescence measurements

[000107] ll fluorescence measurements were performed in 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$. Fluorescence spectra were recorded on Aminco Bowman Series 2 spectrofluorometer (Spectronic Instruments, Rochester, NY). Spectra were corrected for buffer and instrument response. Fluorescence in microplates was read with Tecan Spectra FluorPlus microplate reader (Research Triangle Park, NC). Alternatively, microplates were imaged on Molecular Imager FX (BioRad, Hercules, CA) and fluorescence intensity was determined by integrating the areas of images corresponding to individual wells using QuantityOne software (BioRad). Experiments in 96-well plates and 384-well plates were conducted in 100 µl and 20 µl volume, respectively. Depending on particular instrumentation, slightly different beacon signal changes are recorded due to different buffer background readings with different instruments (depending on the sensitivity of the intrumentation) and different wavelengths of excitation and emission available with each instrument.

[000108] Time-resolved fluorescence in the case of europium chelate – Cy5 labeled beacons was recorded on a laboratory-built instrumentation (Heyduk, T.; Heyduk, E. *Analytical Biochemistry* **2001**, 289, 60-67) which employed pulsed nitrogen laser as the excitation source. Emission was integrated for 100 μs with 30 μsec delay after laser pulse.

Competition assay to determine thrombin aptamer dissociation constants.

[000109] Fluorescence intensity of THR2 in the presence and absence of the competitor was determined. Concentration of thrombin, THR2, and the competitor (when present) were 150 nM, 200 nM, and 200 nM, respectively. Under these conditions, binding of aptamers to thrombin was essentially stoichiometric. Previously described method (Matlock, D.L.; Heyduk, T. *Biochemistry* **2000**, 39, 12274-12283) was used to calculate the ratio of the dissociation constant for THR2 to that of the competitor under these experimental conditions.

Thrombin aptamer binding by electrophoretic mobility shift analysis (EMSA).

[000110] Five microliter samples of 417 nM THR7 were incubated with various amounts of thrombin (0 to 833 nM). After 15 min incubation, 1 μl of 30% Ficoll were added and the samples were run on a 10% polyacrylamide gel in TBE buffer. After the run the gel was stained for 30 min with Sybr Green and the image of the gel was obtained using Molecular Imager FX (BioRad). Intensity of the bands in the gel was determined by integrating the areas of image corresponding to individual bands using QuantityOne software (BioRad).

Design of aptamer-based molecular beacons

[000111] Fig. 17B illustrates the overall concept of molecular beacons for proteins lacking natural sequence-specific DNA binding activity. This design shares some general similarities with molecular beacons for DNA binding proteins described previously by inventor (Heyduk, T.; Heyduk, E. *Nature Biotechnology* **2002**, 20, 171-176; Heyduk, E.; Knoll, E.; Heyduk, T. *Analyt. Biochem.* **2003**, 316, 1-10; Knoll, E.; Heyduk, T. *Analyt. Chem.* **2004**, 76, 1156-1164; Heyduk, E.; Fei, Y.; Heyduk, T. *Combinatorial Chemistry and High-throughput Screening* **2003**, 6, 183-194), (Fig. 17A). Instead of splitting the DNA duplex containing the natural binding site for a protein into the two "half-sites", two aptamers recognizing two nonoverlapping epitopes of the protein are used as functional equivalents of the "half-sites". Short complementary "signaling" oligonucleotides containing the fluorophore and the quencher are attached to the two aptamers via flexible linkers (Fig. 17B). In the absence of the target protein the two-aptamer "half-sites" can not associate since the complementary oligonucleotides are too short to promote efficient annealing. Binding of the aptamer "half-sites" to the target protein brings the two "signaling" oligonucleotides to relative proximity

increasing their local concentrations. This results in the annealing of the "signaling" oligonucleotides, which brings the fluorophore and the quencher to close proximity resulting in a change of fluorescence signal.

Properties of bivalent thrombin aptamers

[000112] We used thrombin as a model system to provide "proof-of-principle" verification of the concept illustrated in Fig. 17B. Thrombin is a proteolytic enzyme involved in blood clotting cascade and naturally does not bind to DNA or RNA. Two laboratories have previously developed DNA aptamers, which selectively recognized two distinct epitopes of the protein (Bock, L.C.; Griffin, L.C.; Latham, J.A.; Vermass, E.H.; Toole, J.J. *Nature* **1992**, 355, 564-566, Tasset, D.M.; Kubik, M.F.; Steiner, W. *J. Mol. Biol.* **1997**, 272, 688-698). One aptamer (G15D; THR4, Table 1) was shown to bind to the heparin-binding exosite (Bock, 1992) whereas the other (60-18 [29]; THR3, Table 1) was shown to bind to fibrinogen-binding exosite (Tasset 1997). As a first step towards developing a beacon recognizing thrombin, we have prepared various aptamer constructs in which the above aptamers were covalently linked by flexible linkers. The primary purpose of these experiments was to determine if linking the two aptamers recognizing two distinct epitopes on protein surface with a flexible linker would produce a bivalent aptamer capable of binding the protein with higher affinity compared to individual aptamers. . This property of such bivalent aptamer constructs is an essential condition necessary for the assay illustrated in Fig. 17B to work. It was essential to experimentally address this question since it is impossible to predict the effect of long flexible linkers on the affinity of these bivalent constructs. A second purpose of these experiments was to establish a suitable length of the linker and the appropriate orientation of 5' and 3' ends of the two aptamers with respect to the linker.

[000113] Individual aptamers were labeled with fluorescein (THR1 (Table 1), specific for fibrinogen-binding exosite, and THR2 (Table 1), specific for heparin-binding exosite) to facilitate determination of the affinity of various constructs for thrombin. Formation of a complex between thrombin and fluorescein-labeled 60-18 [29] aptamer (THR1) could be conveniently followed by fluorescence polarization (not shown) whereas binding of the fluorescein-labeled G15D aptamer (THR2) could be followed by changes in fluorescence intensity (Fig. 18A). Both aptamers bound thrombin in nanomolar concentration range (data not shown for THR1 and Fig. 18A). Quantitative analysis of the binding in the case of THR2 (Fig. 18A) returned the value of K_d of 6.3 nM. This is somewhat higher affinity then previously suggested (Bock 1992, Tasset 1997) which is probably because we used a true equilibrium-binding assay whereas non-equilibrium methodology was used previously. When the binding of THR2 was performed in 10x excess of unlabeled 60-18 [29] aptamer (THR3)

(Fig. 18B) only a small, insignificant decrease in affinity was observed (K_d was 17.7 nM). This confirmed that, as reported previously, G15D and 60-18 [29] aptamers bound independently to two distinct epitopes of thrombin.

[000114] In the next step, the ability of various aptamer constructs to compete with THR2 for binding to thrombin was evaluated. Fluorescence intensity change of THR2 upon addition of thrombin in the presence and absence of the competitor was measured and the amount of THR2 bound to thrombin in the presence of the competitor was calculated as described in Materials and Methods. No aptamer-aptamer interactions could be detected by fluorescence polarization assay (not shown) at aptamer concentrations used in these experiments indicating that the competition data correctly reported on the relative affinity of THR2 and the competitor for binding to thrombin. THR3 was not a competitor (Fig. 18C) in agreement with the data shown in Fig. 18 A and B. THR4 (unlabeled variant of THR2), as expected, was able to compete (Fig. 18C). Quantitative analysis of the competition in this case showed that THR4 bound thrombin 1.7 times better then THR2 indicating that labeling this aptamer with fluorescein had small (insignificant) negative effect on aptamer binding to thrombin. It is obvious that all of the bivalent aptamer constructs were by far better competitors than THR4 (Fig. 18C). THR7 appeared to be the best competitor, essentially completely blocking THR2 binding at 1:1 ratio. Quantitative analysis of the competition in this case revealed that THR7 bound thrombin at least 65 fold tighter then THR2 (estimated K_d for THR7 was < 97 pM). The data shown in Fig. 18C confirmed the expectation that linking two aptamers recognizing two different epitopes of the protein with flexible linkers would produce high-affinity thrombin ligands. Additionally, these data showed that linking the two aptamers by a longer linker (containing 10 Spacer18 units vs. 5 Spacer18) produced slightly better affinity for thrombin (compare binding of THR5 vs. THR6). Also, these data showed that orientation of the aptamers with respect to the linker as in THR7 produced better affinity (compare affinity of THR6 vs. THR7). Thus, in all subsequent constructs, aptamer orientation as in THR7 was used.

[000115] The complex between the bivalent aptamer construct (THR7) and thrombin was stable enough to survive electrophoresis in native polyacrylamide gel (Fig. 18D). We took advantage of this observation and determined stoichiometry of the complex using electrophoretic mobility shift assay (EMSA) (Fried, M. G.; Crothers, D. M. *Nucleic Acid Res.* **1981**, 9, 6505-6525) to follow THR7-thrombin complex formation. We performed a titration of THR7 with thrombin at high concentrations of both molecules. Under these conditions the binding should be stoichiometric. The plot of the complex formed vs. the ratio of thrombin to THR7 indicated 1:1 stoichiometry of the complex (Fig. 18D) consistent with the notion that

both aptamers – components of THR7 – bind to their respective epitopes in THR7-thrombin complex.

Aptamer-based molecular beacon detecting thrombin

[000116] Experimental data described above provided evidence that all necessary conditions for successful implementation of the design of the signaling beacon shown in Fig. 17B were met. Based on these data we have designed thrombin beacon illustrated in Fig. 19A. Thrombin aptamers were connected using 5 Spacer18 linkers to a 7 nucleotide ("nt") complementary oligonucleotides labeled at 5' and 3' with fluorescein and dabcyl, respectively. Mixture of these two constructs bound thrombin much more tightly (~ 36 times) compared to individual aptamers (Fig. 18C) in agreement with high affinity thrombin binding observed for bivalent aptamer constructs in which the two aptamers were permanently linked with a flexible linker. Addition of thrombin to a mixture of fluorochrome and quencher-labeled THR20 and THR21 resulted in protein concentration-dependent quenching of fluorescence intensity (Fig. 19C). Maximum quenching observed was ~ 40%. No fluorescence change was observed (Fig. 19C) upon addition of thrombin to THR21 in the absence of dabcyl-labeled partner (THR20) indicating that fluorescence quenching occurred due to protein-induced increased proximity of signaling oligonucleotides resulting in their annealing as illustrated in Fig. 19B. At nanomolar concentrations of the beacon components and thrombin ~ 15 min of incubation was sufficient to produce maximal response of the beacon. We have also tested thrombin beacons analogous to one shown in Fig. 19 but in which 17 nt poly dT linkers were used in place of Spacer18 linkers. While thrombin-dependent quenching of fluorescence was observed, the quenching was ~ 2 times smaller than with the construct containing Spacer18 linkers. It is likely that poly dT linkers, while flexible, exhibited some residual rigidity (Mills, J.B.; Vacano, E.; Hagerman, P.J. *J. Mol. Biol.* **1999**, 285, 245-257), which perhaps might impede association of the signaling duplex when the two aptamers are bound to thrombin. When the beacon shown in Fig. 19 was titrated with trypsin, a proteolytic enzyme structurally similar to thrombin, no change of fluorescence intensity was observed. We concluded that a functional thrombin beacon according to the design illustrated in Fig. 17B was obtained.

Improvements in beacon performance

[000117] In the next set of experiments, we sought to improve the performance of the beacon by using alternative donor-acceptor label pairs. It has been shown previously that in assays employing FRET as the readout, enhancement of acceptor emission provides potentially better signal to background ratio, higher dynamic range, and better sensitivity (Heyduk, E.; Knoll, E.; Heyduk, T. *Analyt. Biochem.* **2003**, 316, 1-10). We have prepared a series of thrombin beacon constructs analogous to the one depicted in Fig. 17B, but in which various

combinations of fluorescent donor and fluorescent acceptor were incorporated into the signaling oligonucleotides in place of fluorescein-dabcyl pair. THR21 (or THR28 labeled with appropriate NHS ester of the dye) and THR27 labeled with appropriate NHS ester of the dye were used to prepare these beacons. Fig. 20 shows fluorescence spectra of beacons (without and with thrombin addition) labeled with: fluorescein-Texas Red (Fig. 20B), fluorescein-Cy5 (Fig, 20C) and Cy3-Cy5 (Fig. 20D). In all cases functional beacons were obtained. In each case of the beacon with fluorescent donor and fluorescent acceptor, a large thrombin concentration-dependent increase of sensitized acceptor emission was observed (Fig. 20, insets and Fig. 21 A-D). For comparison, Fig. 20A illustrates fluorescence quenching observed in the presence of thrombin in the case of fluorophore-quencher pair (fluorescein-dabcyl). Fig. 21E illustrates results obtained with europium chelate-Cy5 donor-acceptor pair which allowed the use of time-resolved FRET (TR-FRET) as a detection method (Selvin, P.R.; Rana, T.M.; Hearst, J.E. *J. Am. Chem. Soc.* **1994**, 116, 6029-6030; Selvin, P.R.; Hearst, J.E. *Proc. Natl. Acad. Sci USA* **1994**, 91, 10024-10028; Matthis, G. *Clinic. Chem.* **1995**, 41, 1391-1397). With TR-FRET it is possible to eliminate background due to light scattering and prompt fluorescence of directly excited acceptor to further improve signal-to-background ratio of the beacon. Fig. 21F summarizes the performance of beacon variants with various combinations of donor and acceptor probes. The figure shows the fold of signal change in the presence of saturating concentrations of thrombin compared to background signal of the beacon observed in the absence of the protein. This ratio varied from ~ 2 in the case of fluorescein-dabcyl pair to ~ 22 in the case of europium chelate-Cy5 pair. Thus, a substantial improvement of beacon performance can be obtained by selecting optimal donor-acceptor pair and using sensitized acceptor emission as the mode of signal detection. Additional advantage of beacon variants with fluorescent donor and fluorescent acceptor is that their response can be measured by a two-color determination of the ratio of acceptor to donor signals. Such ratiometric measurement provides more stable signal, which is more resistant to nonspecific effects due to light absorption, light scattering or fluorescence quenching caused by additives present in the sample. Increased signal-to-background ratio obtained with optimized donor-acceptor pairs resulted in an increased sensitivity of the beacon. This is illustrated in Fig. 22 which shows responses of three selected beacon variants to low concentrations of thrombin. In the case of fluorescein-dabcyl labeled beacon (the lowest (~ 2 fold) signal change in the presence of saturating concentration of thrombin), statistically significant signal change could only be detected at the highest thrombin concentration tested (1 nM). In the case of fluorescein-Texas Red labeled beacon (~ 5 fold signal change at saturating thrombin concentration), statistically significant signal change could be detected at lower thrombin concentration (200 pM). In the case of fluorescein-Cy5 labeled beacon (~ 15 fold signal change at saturating thrombin concentration), statistically

significant signal change could be detected already at the lowest thrombin concentration tested (50 pM).

[000118] Fig. 23 illustrates excellent reproducibility and stability of thrombin beacon signal. Beacon signal was measured at four thrombin concentrations in five independent measurements. Coefficients of variation were small at each protein concentration tested (Fig. 23A). Beacon signal was stable for at least 24 hours (Fig. 23B).

[000119] Coincidence of three molecular contacts is required to generate a signal with the beacon illustrated in Fig. 17B: two contacts between each of the aptamers and the protein and the contact between the two complementary "signaling" oligonucleotides. Each of these contacts provides its own free energy contribution to the overall stability of beacon-protein complex. Due to exponential relationship between the free energy and equilibrium dissociation constant of the complex, the overall stability of the complex will be greatly decreased if any of the above three molecular contacts would be missing. Thus, it is expected that molecular beacons described here should exhibit greater specificity of protein detection compared to an assay based on a single molecular contact (for example, a single aptamer-based assay). To illustrate this concept we have compared response of a single thrombin aptamer and thrombin beacon to SSB (Single Stranded DNA binding protein from *E. coli*), a protein exhibiting high nonspecific affinity for binding ss DNA (data not shown). SSB at nanomolar concentrations produced a large signal (as measured by fluorescence polarization assay) with the single, fluorescein-labeled aptamer (THR1, Table 1). SSB produced the response in a concentration range very similar to the concentration of thrombin required to bind this aptamer. Thus, single thrombin aptamer exhibited very poor discrimination between SSB and thrombin. In contrast, exposure of thrombin beacon to nanomolar SSB concentration did not produce any significant beacon response, while thrombin at the same concentration range produces large beacon response. Thus, thrombin beacon exhibited excellent discrimination between SSB and thrombin illustrating enhanced specificity of the beacon.

[000120] The primary application of the assay design described here will be in homogeneous high-throughput protein detection. Zhang et al. *Biomol. Screening* **1999,** 4, 67-73) developed a simple statistical parameter, which could be used to evaluate assay for the use in a high-throughput manner. Z'-factor is calculated from large number of repeats of the measurement in the absence and the presence of the protein. Z' value of 1 indicates an ideal assay, Z' value of 0.5 to 1 indicates excellent assay. Z' values below 0.5 indicate assay not well suited for high-throughput applications. Z' value for the thrombin beacon was 0.94 (Fig. 24) which shows that it will be an outstanding high-throughput assay.

Detection of thrombin in complex mixtures

[000121] The next series of experiments addressed the specificity of thrombin beacon and its ability to detect thrombin in cell extracts and in plasma. Response of the beacon to 1 nM thrombin was not affected by 100 and 1000 fold excess of unrelated protein (ovalbumin, Fig. 25A). Also, 100-fold excess of factor Xa, another clotting protease structurally similar to thrombin, did not affect beacon response to 1 nM thrombin (Fig. 25A). A 1000-fold excess of factor Xa attenuated slightly the beacon response but 1 nM thrombin was still readily detectable under these conditions (Fig. 25A). Ovalbumin and factor Xa up to 1 μM concentration had no effect on beacon signal in the absence of thrombin (Fig. 25A). We concluded that the beacon was highly selective for thrombin.

[000122] To test if the beacon could detect thrombin in a complex mixture, we spiked HeLa cellular extract with varying amounts of thrombin and determined beacon response to this mixture (Fig. 25B). Low nanomolar concentrations of thrombin were readily detected. A total of 8 μg of protein were added to 20 μl assay which is within a typical range used in experiments with cellular extracts. Signal observed upon addition of cell extract could be completely abrogated by addition of a specific competitor (unlabeled thrombin aptamer) confirming that the observed signal in the cell extract was due to thrombin. One difficulty we've encountered working with cellular extracts was the degradation of oligonucleotides – components of the assay – by nucleases present in cellular extracts. We have tested various buffer additives to find conditions in which thrombin beacon would remain stable in the presence of cell extracts for sufficiently long period of time. We found that addition of high concentrations of random sequence 30 bp ds DNA (10 μM), high concentrations of 20 nt random sequence ss DNA (0.1μM), and 2.5 mM EGTA protected the thrombin beacon from degradation in the presence of cellular extracts without significantly affecting the response of the beacon to thrombin. Data shown in Fig. 25B were obtained in the presence of the above additives.

[000123] Since thrombin is a plasma protein, we determined if the beacon could be used to detect the protein in plasma. All of the thrombin in plasma is present in a form of its precursor, prothrombin, which is converted to thrombin via proteolytic processing by factor Xa. Prothrombin was recognized by thrombin beacon albeit with much reduced (>20 fold) sensitivity compared to thrombin (not shown). This is well illustrated by experiment shown in Fig. 25C in which sensitized acceptor emission of the beacon in the presence of prothrombin was monitored as a function of time. At the point marked by the arrow Factor Xa was added to the mixture to initiate conversion of prothrombin to thrombin. This conversion resulted in time-dependent increase of beacon signal consistent with a much higher sensitivity of the

beacon to thrombin. Thus, in order to detect thrombin in plasma, factor Xa was included in the assay mixture (Fig. 25D). Adding increasing amounts of plasma resulted in a proportional increase of beacon signal (Fig. 25D). Addition of plasma produced a response of the beacon only if factor Xa was present in the assay. Signal observed upon addition of plasma could be completely abrogated by addition of a specific competitor (unlabeled thrombin aptamer) confirming that the observed signal in the cell extract was due to thrombin. A 5 nL sample of plasma produced a measurable response of the beacon in 20 μl reaction volume. In summary, the experiments illustrated in Fig. 25 demonstrated functionality of thrombin beacon for detecting the protein in complex biological mixtures.

Discussion

[000124] The design of aptamer-based molecular beacons described here is a generalization of the previously developed by us molecular beacons for detecting sequence-specific DNA binding proteins (Fig. 17). Experiments with thrombin as a model protein presented here provided a proof-of-principle evidence for the feasibility of this design. We believe this design will have several important advantages. Since the design of molecular beacons described here is not limited to any specific protein, it will be generally applicable to a large number of proteins. Signaling in the presence of the target protein by our beacon requires a cooperative recognition of two separate epitopes of the protein by two distinct aptamers. This will result in an enhanced specificity of the beacon and increased affinity (i.e. sensitivity of detection). This cooperative action of two aptamers will also allow the use of aptamers with modest affinity to produce molecular beacons binding to target proteins with high affinity and specificity. Aptamers - components of the beacon, do not require any engineering of their structure to tailor their distribution of conformations to allow "switching" between different states in the presence of the protein. Such engineering could be dependent on a particular sequence (structure) of the aptamer and, such balancing of the energetics of alternative conformations of nucleic acids is not necessarily a trivial matter. Since the signaling elements ("signaling" oligonucleotides) in the instant beacon design are separate from its aptamer components, any aptamer sequence (and structure) should be compatible with our beacon design. It is also unlikely that the addition of the "signaling" oligonucleotides will have any deleterious effect on the affinity and specificity of aptamers – components of the beacon. Thus, any protein for which it will be possible to obtain two aptamers recognizing two distinct epitopes of the protein should be a good target for developing molecular beacons according to scheme in Fig.17.

[000125] Antibodies recognizing distinct epitopes of the protein can be obtained relatively easily. Similarly, there are no reasons why aptamers recognizing distinct epitopes could not be

developed for many target proteins and several examples are already available (Jayasena, S.D. *Clinical Chem.* **1999**, 45, 1628-1650). Several approaches towards achieving this goal would be possible. The first approach would be to perform *in vitro* selections (SELEX) using different methods for separation of protein-bound and unbound oligonucleotides. The rationale here is that in these different partitioning methods different regions of the protein could be preferentially displayed resulting in aptamers directed to different regions of protein surface. Aptamers selected to thrombin are an example of such approach (Bock, 1992; Tasset, 1997). The second approach could be to raise the aptamers to peptides corresponding to different regions of the target protein molecule. Experimental evidence exists to show that such strategy can be used to develop aptamers capable of recognizing the intact protein from which the peptide used as a target for aptamer development was derived (Wei, X.; Ellington, A.D. *Proc. Natl. Acad. Sci. USA* **1996**, 93, 7475-7480). Such approach is widely used to generate antibodies recognizing proteins. Two aptamers recognizing different epitopes of the protein can be also produced by a two-step sequential SELEX in which the second step involves selecting an aptamer in the presence of saturating concentration of the aptamer selected in the first step. We have validated this procedure using thrombin as a model system (Heyduk, E. and Heyduk, T., unpublished). Finally, we have developed a novel *in vitro* selection strategy to produce pairs of aptamers specifically designed to function in our molecular beacon design (Heyduk, E., Kalucka, J., Kinnear, B., Knoll, E., and Heyduk, T., unpublished). Thus, multiple routes to obtain pairs of aptamers recognizing non-overlapping epitopes of the protein will available.

EXAMPLE 4: SENSOR DESIGN VARIATIONS

[000126] Several variations of the instant molecular beacon are applicable in the practice of this invention. Those variants of the sensor design are depicted in Figure 26 and summarized herein (supra). The sensor design depicted in Fig. 26F is demonstrated to effectively detect DNA binding proteins. Upon the titration of cAMP response element binding protein ("CRP"), which is an example of a DNA binding protein, to a mixture of donor and acceptor labeled sensor components, there is a concomitant increase in sensitized acceptor fluorescence intensity (Figure 27).

[000127] The sensor design depicted shown in Fig. 26G is demonstrated in Figure 28. Panel A depicts the principle of the sensor function. Upon the addition of single stranded DNA, which contains two distinct sequence elements that are complementary to elements in the sensor, to the mixture of two donor and acceptor labeled sensor components, there is a concomitant increase in sensitized acceptor fluorescence intensity (Figure 28, B, line with + sign). The sensor in this particular case contained Texas Red-labeled THR29 and THR32.

[000128] The increased specificity of the instant molecular beacon sensor design compared to assays based on a single, target macromolecule-recognizing element was experimentally demonstrated (Figure 29). Recognition of the target molecule by the sensor involves coincidence of three molecular contacts each providing a free energy (ΔG) contribution to the overall stability of the complex. Due to exponential relationship between the free energy and equilibrium dissociation constant of the complex, the overall stability of the complex will be greatly decreased if any of the above three molecular contacts would be missing resulting in high specificity of target molecule recognition. A nonspecific single stranded DNA binding protein("SSB") at nanomolar concentrations produced a large signal (as measured by fluorescence polarization assay) with the single, fluorescein-labeled aptamer (THR1, Table 1). SSB produced the response in a concentration range very similar to the concentration of thrombin required to bind this aptamer. Thus, a single thrombin aptamer exhibited very poor discrimination between SSB and thrombin. (Panel B) Exposure of the thrombin sensor (a mixture of THR21 (fluorescein-labeled) and Texas Red labeled THR27) to nanomolar SSB concentration did not produce any significant beacon response (dashed lines), while thrombin at the same concentration range produces large beacon response.(Panel C). Thus, the thrombin beacon exhibited excellent discrimination between SSB and thrombin, illustrating the enhanced specificity of the beacon.

Methods for preparing aptamers for the variant sensors

[000129] Figure 30 summarizes a method for selecting aptamers useful in the practice of the invention. Panel A depicts the selection of a second aptamer in the presence of the protein bound to the first aptamer. A signaling oligo is at the 5'-end of the random-sequence containing construct and the complementary signaling oligo is attached to the first aptamer via a long flexible linker. Selection of co-aptamers using this type of random DNA (or RNA) construct will be biased towards aptamers which are capable of binding to the protein at a site distinct from the epitope of the first aptamer, and which will function in sensors depicted in Fig. 26A.

[000130] An alternative scenario is depicted in panel B, which describes the simultaneous selection of two aptamers binding two distinct epitopes of the protein. The bars (at the end of primer 1 and primer 4) depict short complementary sequences at the 5'-end and 3'-end of a random-sequence containing the aptamer constructs. Selection of aptamers using such random DNA (or RNA) constructs will be biased towards aptamers that are capable of binding to the protein simultaneously at two distinct epitopes of the protein, and which will function in sensors depicted in Fig. 26A.

[000131] In yet another alternate embodiment, a second aptamer can be selected in the presence of the protein bound with a double stranded DNA (Figure 30, panel C). The bar depicts the short sequence (at the 5'-end of the random-sequence containing construct) complementary to the signaling oligonucleotide attached to the double stranded DNA via a long flexible linker. Selection of co-aptamers using such a random DNA (or RNA) construct will be biased towards aptamers that are capable of binding to the protein at a site distinct from the double stranded DNA binding site of the protein and which will function in sensors depicted in Fig. 17B.

[000132] In yet another alternate embodiment, a second aptamer can be selected in the presence of the protein bound with an antibody at a distinct epitope of the protein(Figure 30, panel D). The bar depicts the short sequence (at the 5'-end of the random-sequence containing construct) complementary to the signaling oligonucleotide attached to the antibody via a long flexible linker. Selection of co-aptamers using such random DNA (or RNA) construct will be biased towards aptamers which would be able to bind to the protein at a site distinct from the epitope of antibody and which will function in sensors depicted in Fig. 17C.

[000133] The selection of an aptamer binding to the thrombin at the epitope distinct from the binding site of G15D aptamer was performed using SELEX procedure starting from a construct containing 33 nt random sequence (THR11) in the presence of the excess of G15D aptamer-containing construct (THR22) (Figure 31, panel A). Panel B depicts the thrombin binding activity of single stranded DNAs obtained after each indicated round of selection. Measurable thrombin binding activity appeared after 4^{th} selection and reached maximum after 12^{th} selection. Binding was measured in the presence of the excess of THR22. DNA obtained after 12^{th} selection was cloned and DNA obtained from the individual clones was sequenced. Panel C depicts the sequence alignment (using ClustalX) of the individual clones. Clones obtained from 4 independent selection experiments are shown. These selections were performed using the following pairs of aptamer constructs and random sequence-containing constructs: THR22 and THR 11; THR25 and THR 11; THR42 and THR11; THR43 and THR 11. Several families of highly conserved sequences are easily visible in panel C.

[000134] A functional thrombin sensor comprising Texas Red-labeled THR27 and fluorescein-labeled THR35 or THR36, which contain sequences corresponding to that of clones 20-26 from Fig, 31C, is depicted in Figure 32. THR35 and THR36 differ by the length of DNA sequence flanking the sequence of clones 20-26. The fluorescence image (sensitized acceptor emission) of wells of a microplate containing 20 nM (panel A) or 100 nM (panel B) of the indicated thrombin sensor and the indicated concentrations of thrombin are shown. For comparison, a sensor comprising THR21 and THR27 is shown.

[000135] Figure 33 summarizes of a simultaneous selection of two aptamers binding to thrombin at two distinct epitopes. Selection of aptamers was performed using the SELEX procedure starting from two constructs containing 30 nt random sequence (THR49 and THR50) (panel A). Thrombin binding activity of the mixture of single stranded DNA's obtained after each indicated round of selection is shown in panel B. Measurable thrombin binding activity appeared after 6^{th} selection and reached a maximum after the 14^{th} selection. DNA obtained after 14^{th} selection was cloned and the DNA obtained from the individual clones were sequenced. Panel C depicts the sequence alignment (using ClustalX) of the clones. Several families of highly conserved sequences are easily visible.

[000136] Aptamer-based molecular beacons were developed for cAMP response element binding protein ("CRP"). Aptamers were selected to bind at sites distinct from the DNA binding site of the protein. Selection was performed using SELEX procedure starting from a construct containing 33 nucleotide random sequence (MIS12) in the presence of the excess of CRP binding site-containing construct (MIS10X3 hybridized with MIS11) (Figure 34, panel A). CRP binding activity of single stranded DNA that was obtained after indicated round of selection is depicted in Figure 34, panel B. Measurable CRP binding activity appeared after 6^{th} selection and reached maximum after 12^{th} selection. Binding was measured in the presence of the excess of MIS10X3 hybridized with MIS11. DNA obtained after 12^{th} selection was cloned and DNA obtained from the individual clones were sequenced. The sequence alignment (using ClustalX) of the clones is depicted in panel C. Conserved core sequence of ~16 nucleotides could be identified.

WHAT IS CLAIMED IS

1. A method of detecting a polypeptide in a sample comprising the steps of (a) contacting a sample with a first aptamer construct and a second aptamer construct, and (b) detecting an association of the first aptamer construct, the second aptamer construct, and a polypeptide by a detection method; wherein (c) the first aptamer construct is capable of binding to a first epitope of the polypeptide and the second aptamer construct is capable of binding to a second epitope of the polypeptide, (d) the first aptamer construct comprises (i) a first aptamer that can bind to the first epitope, (ii) a first signaling oligo and (iii) a first label, and (e) the second aptamer construct comprises (iv) a second aptamer that can bind to the second epitope, (v) a second signaling oligo, which is complementary to the first signaling oligo, and (vi) a second label.

2. The method of claim 1 wherein the first and second signaling oligo each consists of at least 5 nucleotides and no more than 7 nucleotides.

3. The method of claim 1 wherein the first aptamer comprises a natural cognate binding element sequence and the second aptamer is selected using in vitro evolution.

4. The method of claim 1 wherein the first label is a fluorescence donor and the second label is a fluorescence acceptor.

5. The method of claim 1 wherein the first label is a fluorescence acceptor and the second label is a fluorescence donor.

6. The method of claim 1 wherein the detection method detects a change in fluorescence.

7. The method of claim 21 wherein the detection method is FRET.

8. The method of claim 1 wherein the first aptamer and the second aptamer are selected using in vitro evolution.

9. The method of claim 1 wherein the polypeptide does not naturally bind a natural cognate binding element sequence.

10. The method of claim 9 wherein the polypeptide is a thrombin.

11. The method of claim 10 wherein the first aptamer binds to fibrinogen exocite of the thrombin and the second aptamer binds to a fibrinogen exocite of the thrombin.

12. The method of claim 11 wherein the first label is a fluorescein.

13. The method of claim 12 wherein the detection method is fluorescence polarization.

14. The method of claim 12 wherein the second label is a dabcyl and the detection method is a detecting a change in fluorescein fluorescence intensity.

15. The method of claim 1 wherein the first aptamer construct and the second aptamer construct are joined together by a linker.

16. The method of claim 1 wherein the linker is a flexible Spacer 18 linker.

17. A method of detecting an analyte in a sample comprising the steps of (a) contacting a sample with a first aptamer construct, a second aptamer construct, and a polypeptide, and (b) detecting an association of the first aptamer construct, the second aptamer construct, the polypeptide and an analyte by a detection method; wherein (c) in the presence of the analyte, the first aptamer construct is capable of binding to a first epitope of the polypeptide and the second aptamer construct is capable of binding to a second epitope of the polypeptide, and (d) the first aptamer construct comprises a first aptamer that can bind to the first epitope, a first signaling oligo and a first label, and (e) the second aptamer construct comprises a second aptamer that can bind to the second epitope, a second signaling oligo, which is complementary to the first signaling oligo, and a second label.

18. The method of claim 17 wherein the first signaling oligo and second signaling oligo each consists of at least 5 nucleotides and no more than 7 nucleotides.

19. The method of claim 17 wherein the first aptamer comprises a natural cognate binding element sequence and the second aptamer is selected using in vitro evolution.

20. The method of claim 17 wherein the first label is a fluorescence donor and the second label is a fluorescence acceptor.

21. The method of claim 17 wherein the first label is a fluorescence acceptor and the second label is a fluorescence donor.

22. The method of claim 17 wherein the detection method detects a change in fluorescence.

23. The method of claim 22 wherein the detection method is FRET.

24. The method of claim 17 wherein the first aptamer and the second aptamer are selected using in vitro evolution.

25. The method of claim 17 wherein the polypeptide does not naturally bind a natural cognate binding element sequence.

26. The method of claim 17 wherein the polypeptide undergoes a conformational change upon binding the analyte.

27. The method of claim 26 wherein the analyte is a drug and the polypeptide is capable of binding the drug.

28. The method of claim 27 wherein the analyte is a statin drug and the polypeptide is a HMG-CoA reductase.

29. The method of claim 17 wherein the analyte is a toxin found in the environment.

30. The method of claim 17 wherein the first aptamer construct and the second aptamer construct are joined together by a linker.

31. The method of claim 30 wherein the linker is a flexible Spacer 18 linker.

32. A method of making a set of aptamer constructs, comprising a first and second aptamer construct, comprising the steps of (a) selecting a first aptamer against a first substrate, which comprises a first epitope, and selecting a second aptamer against a second substrate, which comprises a second epitope, wherein the first aptamer is capable of binding to the first epitope and the second aptamer is capable of binding to the second epitope, (b) attaching a first label to the first aptamer and attaching a second label to the second aptamer, (c) attaching a first signaling oligo to the first aptamer and attaching a second signaling oligp to the second aptamer, wherein the second signaling oligo is complementary to the first signaling oligo, and (d) such that (i) the first aptamer construct comprises the first aptamer, the first label and the first signaling oligo, and (ii) the second aptamer construct comprises the second aptamer, the second label and the second signaling oligo.

33. The method of claim 32 wherein the first substrate is a polypeptide and the second substrate is the polypeptide bound to the first aptamer, wherein the first aptamer masks the first epitope.

34. The method of claim 32 wherein the first substrate is a polypeptide or a macromolecular complex and the second substrate is the polypeptide or macromolecular complex bound to the first aptamer, wherein the first aptamer (a) is attached to the first signaling oligo or the first label and (b) masks the first epitope.

35. The method of claim 32 wherein the first substrate is a fragment of a polypeptide consisting essentially of the first epitope and the second substrate is a fragment of the polypeptide consisting essentially of the second epitope.

36. The method of claim 32 wherein the first signaling oligo and the second signaling oligo each consist of at least 5 nucleotides and no more than 7 nucleotides.

37. The method of claim 32 wherein the first aptamer comprises a natural cognate binding element sequence and the second aptamer is selected using in vitro evolution.

38. The method of claim 32 wherein the first label is a fluorescence donor and the second label is a fluorescence acceptor.

39. The method of claim 32 wherein the first label is a fluorescence acceptor and the second label is a fluorescence donor.

40. The method of claim 32 wherein the first aptamer and the second aptamer are selected using in vitro evolution.

41. The method of claim 32 comprising the step joining the first aptamer construct to the second aptamer construct with a flexible linker.

42. A bivalent aptamer construct comprising a first aptamer, a first label, a first signaling oligo, a second aptamer, a second label, a second signaling oligo and a linker, wherein the first aptamer is capable of binding to a first epitope and the second aptamer is capable of binding to a second epitope.

43. The bivalent aptamer construct of claim 42 wherein the first epitope and the second epitope are distinct and non-overlapping epitopes of a same polypeptide.

44. The bivalent aptamer construct of claim 42 wherein the linker is a flexible linker.

45. The bivalent aptamer construct of claim 44 wherein the linker is a Spacer 18.

46. The bivalent aptamer construct of claim 44 wherein the linker is a deoxythymidine polymer.

47. The bivalent aptamer construct of claim 42 wherein the first label is a fluorescence donor.

48. The bivalent aptamer construct of claim 47 wherein the second label is a fluorescence recipient.

49. The bivalent aptamer construct of claim 42 wherein the first and second signaling oligos are at least 5 nucleotides in length and no more than 7 nucleotides in length.

50. The bivalent aptamer construct of claim 42 wherein the first aptamer comprises a natural cognate binding element sequence.

51. The bivalent aptamer construct of claim 50 wherein the second aptamer is selected using in vitro evolution.

52. The bivalent aptamer construct of claim 42 wherein the first aptamer is selected using in vitro evolution.

53. The bivalent aptamer construct of claim 42 wherein the polypeptide is a thrombin, the first label is a fluorescein, the second label is a dabcyl, the first epitope is a heparin exosite, the second epitope is a fibrinogen exosite, and the linker is a Spacer 18.

54. The bivalent aptamer construct of claim 43 wherein the polypeptide is a thrombin, the first label is a fluorescein, the second label is a dabcyl, the first epitope is a heparin exosite, the second epitope is a fibrinogen exosite, and the linker is a Spacer 18.

55. A kit comprising a first epitope binding agent, to which is attached a first label, and a second epitope binding agent, to which is attached a second label, wherein (a) when the first epitope binding agent and the second epitope binding agent label bind to a first epitope of a polypeptide and a second epitope of the polypeptide, respectively, (b) the first label and the second label interact to produce a detectable signal.

56. The kit of claim 55 wherein the first epitope binding agent is an antibody.

57. The kit of claim 55 wherein the first epitope binding agent is a first aptamer construct, which comprises a first aptamer, a first label and a first signaling oligo.

58. The kit of claim 57 wherein the second epitope binding agent is a second aptamer construct, which comprises a second label and a second signaling oligo, which is complementary to the first signaling oligo.

59. The kit of claim 58 wherein the first signaling oligo and the second signaling oligo are at least 5 nucleotides in length and no more than 7 nucleotides in length.

60. The kit of claim 58 wherein the first aptamer comprises a natural cognate binding element sequence.

61. The kit of claim 58 wherein the second aptamer was selected using in vitro evolution.

62. The kit of claim 55 wherein the first label is a fluorescence donor and the second label is a fluorescence recipient.

63. The kit of claim 62 wherein the first label is a fluorescein and the second label is a dabcyl.

64. The kit of claim 55 further comprising the polypeptide, wherein the polypeptide is capable of binding an analyte.

65. The kit of claim 55 further comprising a printed set of instructions for using said kit.

66. A method of diagnosing a disease comprising the steps of (a) obtaining a sample from a patient, (b) contacting the sample with a first epitope binding agent and a second epitope binding agent, and (c) detecting the presence of a polypeptide in the sample using a detection method, wherein the presence of the polypeptide in the sample indicates whether a disease is present in the patient.

67. The method of claim 66 wherein (a) the first epitope binding agent is a first aptamer to which a first label and a first signaling oligo are attached, (b) the second epitope binding agent is a second aptamer to which a second label and a second signaling oligo, which is complementary to the first signaling oligo, are attached, and (c) the detection method is a fluorescence detection method, wherein, (d) when the first aptamer binds to the polypeptide and the second aptamer binds to the polypeptide, (e) the first signaling oligo and the second signaling oligo associate with each other, and (f) the first label is brought into proximity to the second label such that a change in fluorescence occurs.

68. The method of claim 66 wherein the sample is selected from the group consisting of blood, urine, ascites and tissue sample.

69. The method of claim 66 wherein the patient is a human.

70. A method of diagnosing a disease comprising the steps of (a) obtaining a sample from a patient, (b) contacting the sample with a first epitope binding agent, a second epitope binding agent, and a polypeptide, which comprises a first epitope and a second epitope, and (c) detecting in the sample, using a detection method, the presence of an analyte, which is capable of binding the polypeptide, wherein the presence of the analyte indicates whether a disease is present in the patient.

71. The method of claim 70 wherein (a) the first epitope binding agent is a first aptamer to which a first label and a first signaling oligo are attached, (b) the second epitope binding agent is a second aptamer to which a second label and a second signaling oligo, which is complementary to the first signaling oligo, are attached, and (c) the detection method is a fluorescence detection method, wherein, (d) when the analyte binds to the polypeptide, (e) the first aptamer binds to the polypeptide and the second aptamer binds to the polypeptide, (e) the first signaling oligo and the

second signaling oligo associate with each other, and (f) the first label is brought into proximity to the second label such that a change in fluorescence occurs.

72. The method of claim 70 wherein the sample is selected from the group consisting of blood, urine, ascites and tissue sample.

73. The method of claim 70 wherein the patient is a human.

74. A method of screening a sample for useful reagents comprising the steps of (a) contacting a sample with a first epitope binding agent and a second epitope binding agent, and (b) detecting the presence of a useful reagent in the sample using a detection method.

75. The method of claim 74 wherein the useful reagent is a polypeptide which comprises a first epitope and a second epitope.

76. The method of claim 74 further comprising the step of contacting the sample with a polypeptide, which is capable of binding an analyte, wherein the useful reagent is an analyte.

77. The method of claim 74 wherein the useful reagent is a potential therapeutic composition.

78. The method of claim 74 wherein (a) the first epitope binding agent is a first aptamer to which a first label and a first signaling oligo are attached, (b) the second epitope binding agent is a second aptamer to which a second label and a second signaling oligo, which is complementary to the first signaling oligo, are attached, and (c) the detection method is a fluorescence detection method, wherein, (d) when the first aptamer binds to the polypeptide and the second aptamer binds to the polypeptide, (e) the first signaling oligo and the second signaling oligo associate with each other, and (f) the first label is brought into proximity to the second label such that a change in fluorescence occurs.

79. The method of claim 78 wherein the useful reagent is a polypeptide which comprises a first epitope and a second epitope.

80. The method of claim 78 further comprising the step of contacting the sample with a polypeptide, which is capable of binding an analyte, wherein the useful reagent is an analyte.

81. The method of claim 78 wherein the useful reagent is a potential therapeutic composition.

82. A pharmaceutical composition comprising a bivalent aptamer construct as set forth in any one of claims 42-54.

83. A method of facilitating molecular interactions in a sample comprising the step of administering to the sample a bivalent aptamer as set forth in any one of claims 42-54, wherein

the first epitope and the second epitope are on separate molecular entities and the first epitope and second epitope are brought into close proximity to effect a molecular interaction.

84. The method of claim 83 wherein the sample is selected from the group consisting of cell, tissue, cerebral spinal fluid, blood, in vitro reaction mixture, and environmental system.

85. A method of detecting a polypeptide in a sample comprising the steps of (a) contacting a sample with a first molecular-recognition construct and a second molecular-recognition construct, and (b) detecting an association of the first molecular-recognition construct, the second molecular-recognition construct, and a polypeptide by a detection method; wherein (c) the first molecular-recognition construct is capable of binding to a first epitope of the polypeptide and the second molecular-recognition construct is capable of binding to a second epitope of the polypeptide, (d) the first molecular-recognition construct comprises (i) a first epitope-binding agent that can bind to the first epitope, (ii) a first signaling oligo and (iii) a first label, and (e) the second molecular-recognition construct comprises (iv) a second epitope binding agent that can bind to the second epitope, (v) a second signaling oligo, which is complementary to the first signaling oligo, and (vi) a second label.

86. The method of claim 85 wherein the first epitope binding agent is an aptamer.

87. The method of claim 86 wherein the second epitope binding agent is an aptamer.

88. The method of claim 86 wherein the second epitope binding agent is an antibody.

89. The method of claim 86 wherein the second epitope binding agent is a double stranded polynucleotide containing binding site for the polypeptide.

90. The method of claim 85 wherein the first epitope binding agent is an antibody.

91. The method of claim 90 wherein the second epitope binding agent is a second antibody.

92. The method of claim 90 wherein the second epitope binding agent is a double stranded polynucleotide containing binding site for the polypeptide.

93. The method of claim 85 wherein the first epitope binding agent is a double stranded polynucleotide containing first binding site for the polypeptide.

94. The method of claim 93 wherein the second epitope binding agent is a double stranded polynucleotide containing second binding site for the polypeptide.

95. The method of any one of claims 85 through 94 wherein the detection method is selected from the group consisting of plasmon resonance, fluorescence resonance energy transfer

("FRET)), FCCS, fluorescence quenching, fluorescence polarization, production of a colored product, chemiluminescence, scintillation, bioluminescence, and luminescence resonance energy transfer.

96. The method of claim 95 wherein the detection method is luminescence resonance energy transfer.

97. The method of any one of claims 85 through 96 wherein the polypeptide is thrombin or cAMP response element binding protein ("CRP").

98. The method of any one of claims 85 through 97 wherein the sample is selected from the group consisting of blood, urine, ascites, cellular sample and tissue sample.

99. A molecular beacon comprising a first molecular-recognition construct and a second molecular-recognition construct; wherein (a) the first molecular-recognition construct is capable of binding to a first epitope of a polypeptide and the second molecular-recognition construct is capable of binding to a second epitope of the polypeptide, (b) the first molecular-recognition construct comprises (i) a first epitope-binding agent that can bind to the first epitope, (ii) a first signaling oligo and (iii) a first label, and (c) the second molecular-recognition construct comprises (iv) a second epitope binding agent that can bind to the second epitope, (v) a second signaling oligo, which is complementary to the first signaling oligo, and (vi) a second label.

100. The molecular beacon of claim 99 wherein the first epitope binding agent is an aptamer.

101. The method of claim 100 wherein the second epitope binding agent is an aptamer.

102. The method of claim 100 wherein the second epitope binding agent is an antibody.

103. The method of claim 100 wherein the second epitope binding agent is a double stranded polynucleotide containing binding site for the polypeptide.

104. The method of claim 99 wherein the first epitope binding agent is an antibody.

105. The method of claim 104 wherein the second epitope binding agent is a second antibody.

106. The method of claim 104 wherein the second epitope binding agent is a double stranded polynucleotide containing binding site for the polypeptide.

107. The method of claim 99 wherein the first epitope binding agent is a double stranded polynucleotide containing first binding site for the polypeptide.

108. The method of claim 107 wherein the second epitope binding agent is a double stranded polynucleotide containing second binding site for the polypeptide.

Figure 1

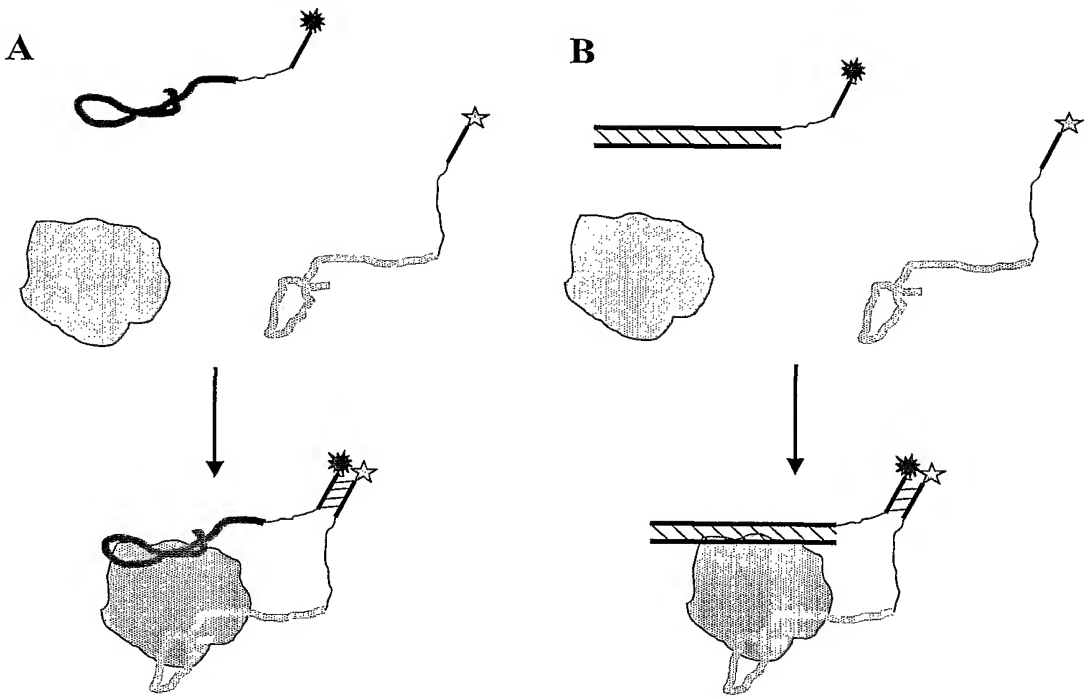


Figure 2

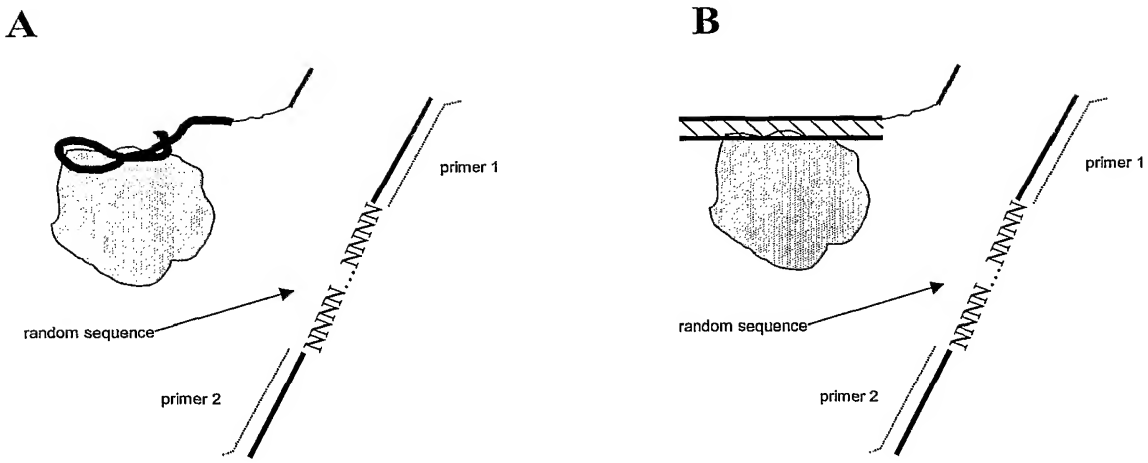

A
primer 1
random sequence
NNNN...NNNN
primer 2
B
primer 1
random sequence
NNNN...NNNN
primer 2

Figure 3

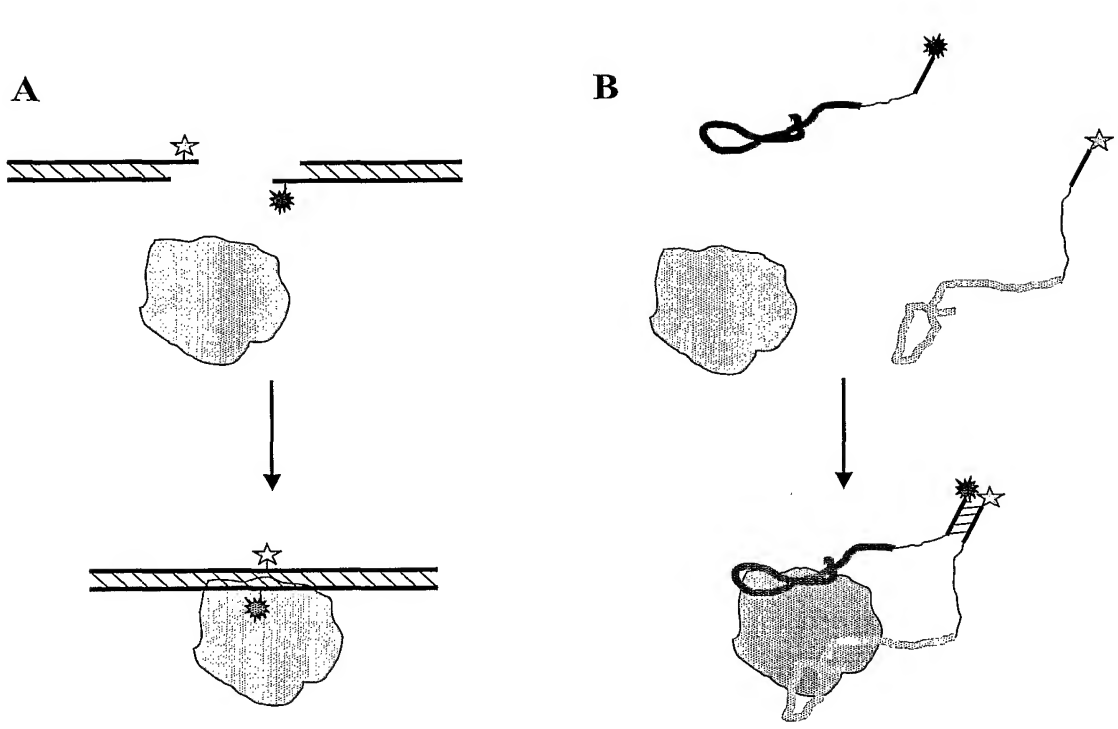


Figure 4

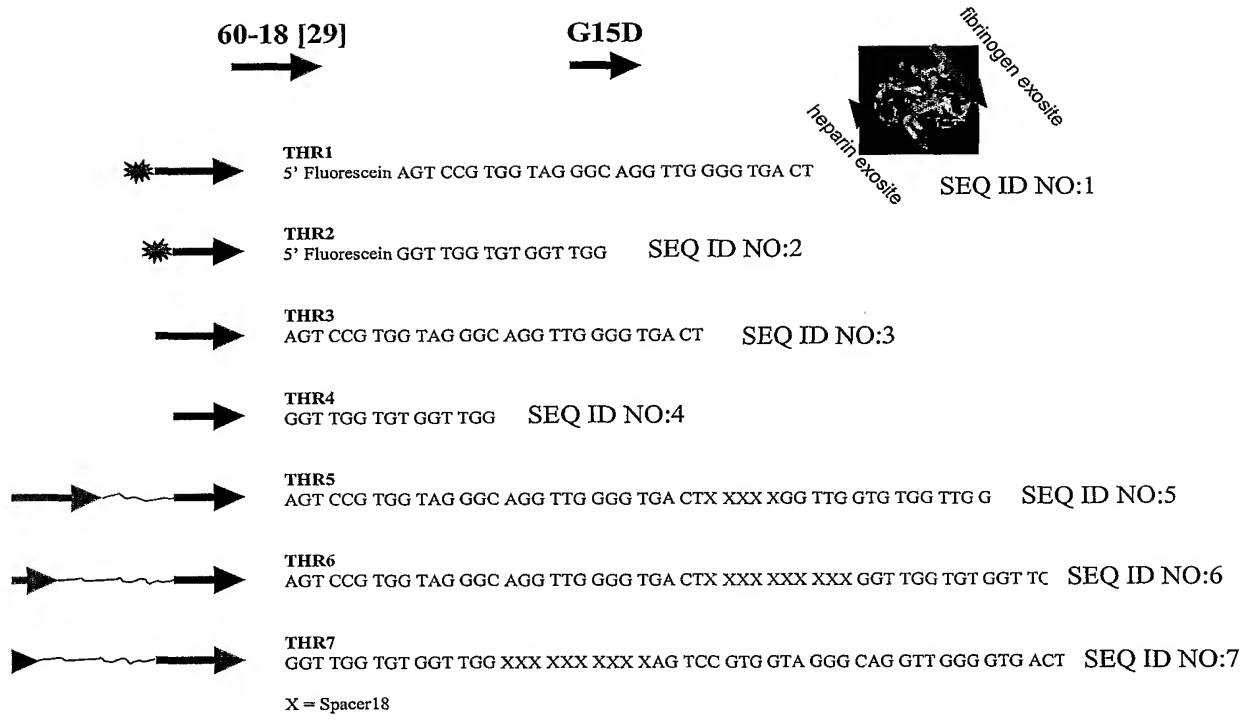

60-18 [29]
G15D
fibrinogen exosite
heparin exosite
THR1
5' Fluorescein AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
SEQ ID NO:1
THR2
5' Fluorescein GGT TGG TGT GGT TGG
SEQ ID NO:2
THR3
AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
SEQ ID NO:3
THR4
GGT TGG TGT GGT TGG
SEQ ID NO:4
THR5
AGT CCG TGG TAG GGC AGG TTG GGG TGA CTX XXX XGG TTG GTG TGG TTG G
SEQ ID NO:5
THR6
AGT CCG TGG TAG GGC AGG TTG GGG TGA CTX XXX XXX XXX GGT TGG TGT GGT TC
SEQ ID NO:6
THR7
GGT TGG TGT GGT TGG XXX XXX XXX XAG TCC GTG GTA GGG CAG GTT GGG GTG ACT
SEQ ID NO:7
X = Spacer18

Figure 5

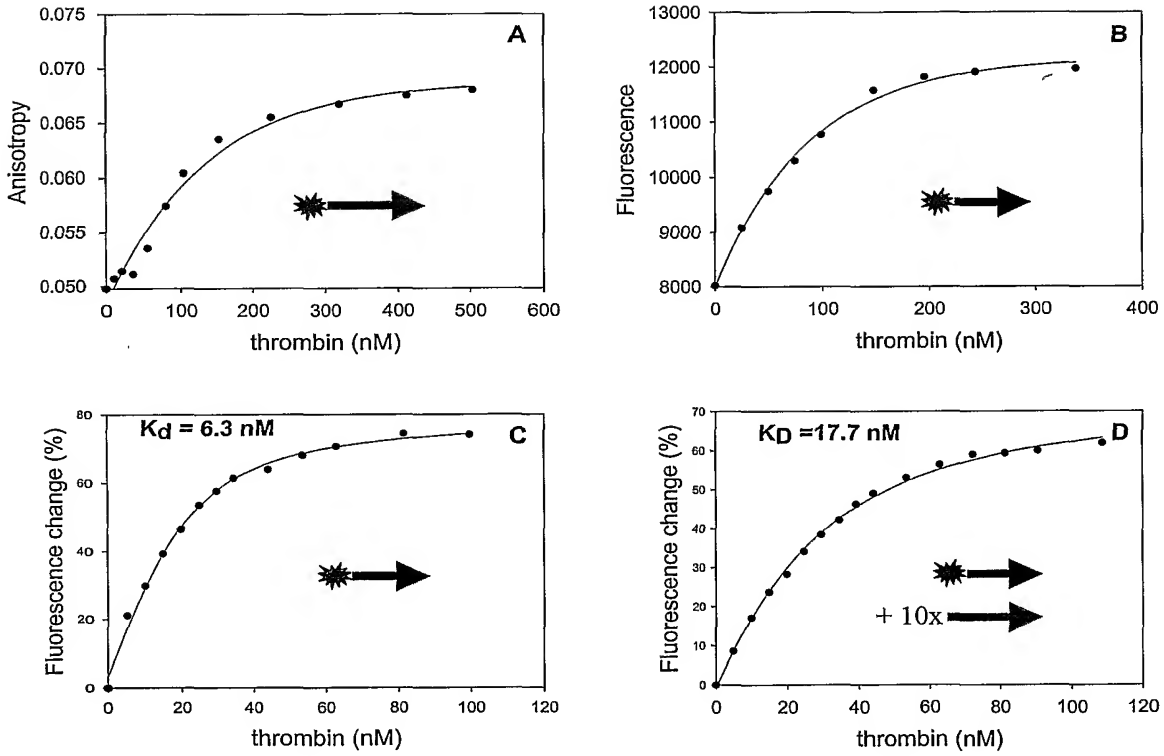

A
Anisotropy
0.075
0.070
0.065
0.060
0.055
0.050
0
100
200
300
400
500
600
thrombin (nM)
B
Fluorescence
13000
12000
11000
10000
9000
8000
0
100
200
300
400
thrombin (nM)
C
Kd = 6.3 nM
Fluorescence change (%)
80
60
40
20
0
0
20
40
60
80
100
120
thrombin (nM)
D
KD =17.7 nM
+ 10x
Fluorescence change (%)
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
thrombin (nM)

Figure 6

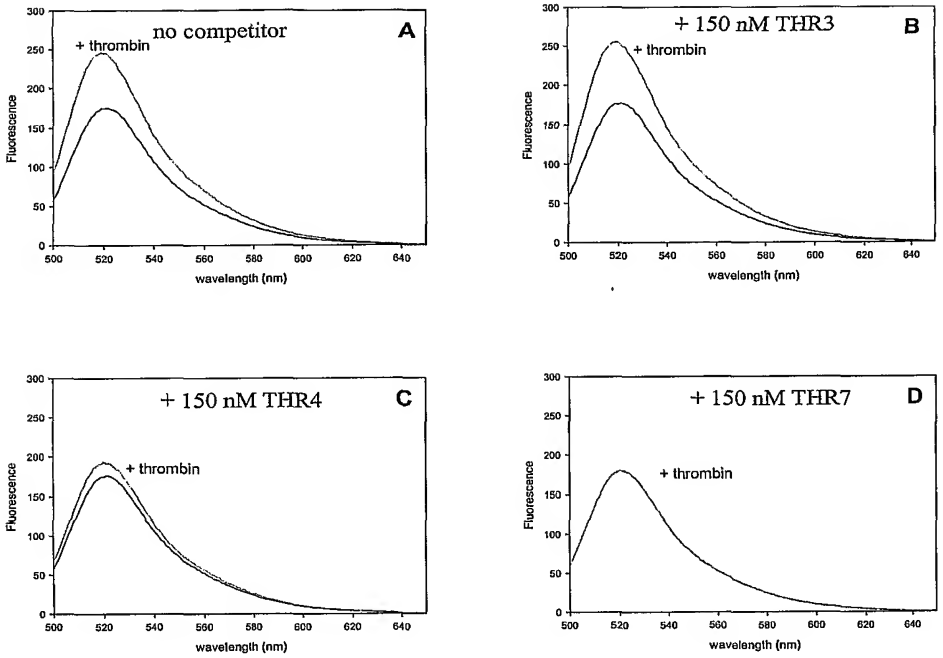

no competitor
A
+ thrombin
+ 150 nM THR3
B
+ thrombin
+ 150 nM THR4
C
+ thrombin
+ 150 nM THR7
D
+ thrombin
Fluorescence
wavelength (nm)
0
50
100
150
200
250
300
500
520
540
560
580
600
620
640

Figure 7

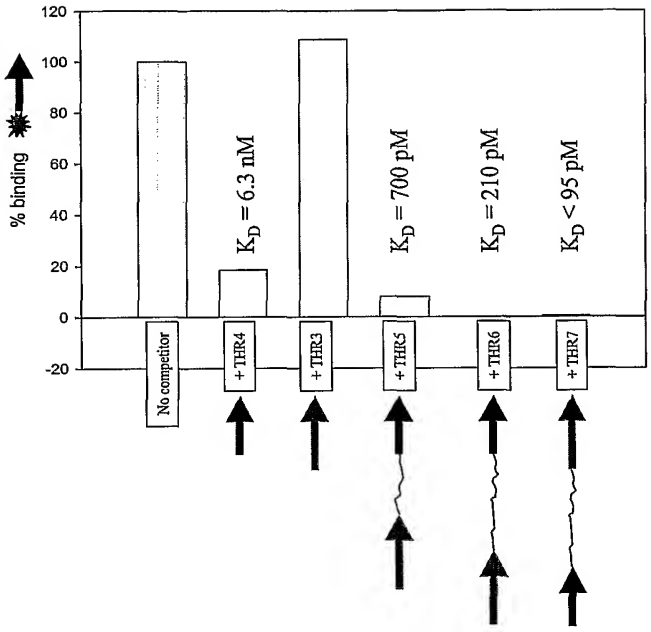

120
100
80
60
40
20
0
-20
% binding
No competitor
+ THR4
+ THR3
+ THR5
+ THR6
+ THR7
K_D = 6.3 nM
K_D = 700 pM
K_D = 210 pM
K_D < 95 pM

Figure 8

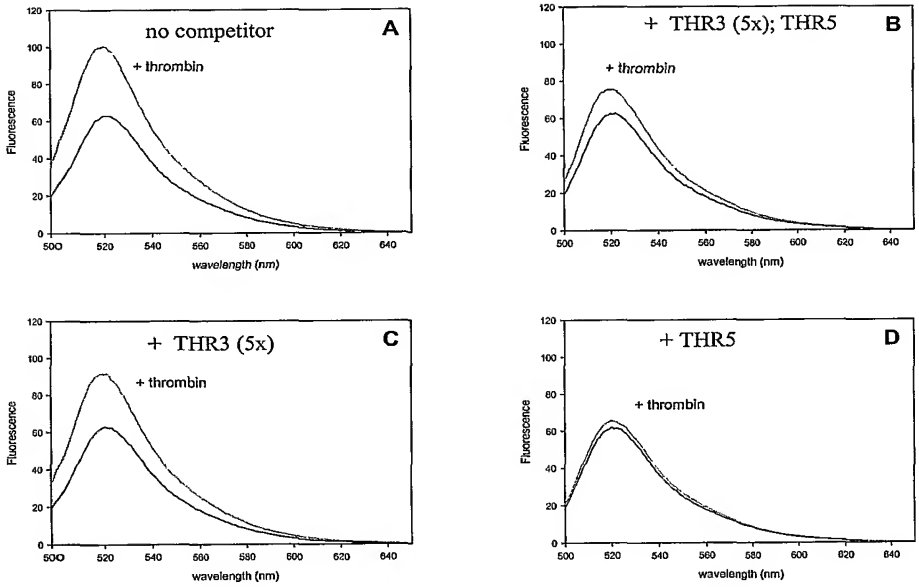

no competitor
A
+ thrombin
Fluorescence
wavelength (nm)
+ THR3 (5x); THR5
B
+ thrombin
Fluorescence
wavelength (nm)
+ THR3 (5x)
C
+ thrombin
Fluorescence
wavelength (nm)
+ THR5
D
+ thrombin
Fluorescence
wavelength (nm)

Figure 9

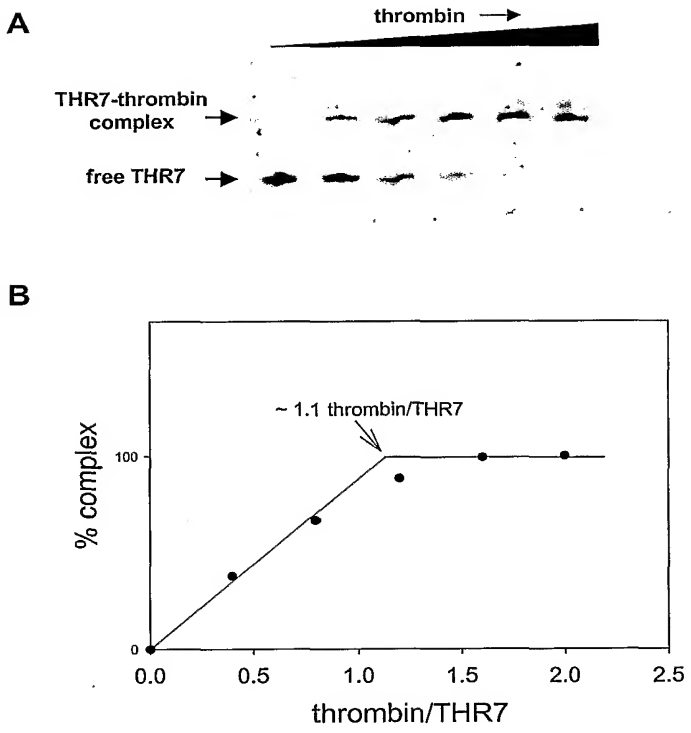

A
thrombin
THR7-thrombin complex
free THR7
B
~ 1.1 thrombin/THR7
% complex
100
0
0.0
0.5
1.0
1.5
2.0
2.5
thrombin/THR7

Figure 10

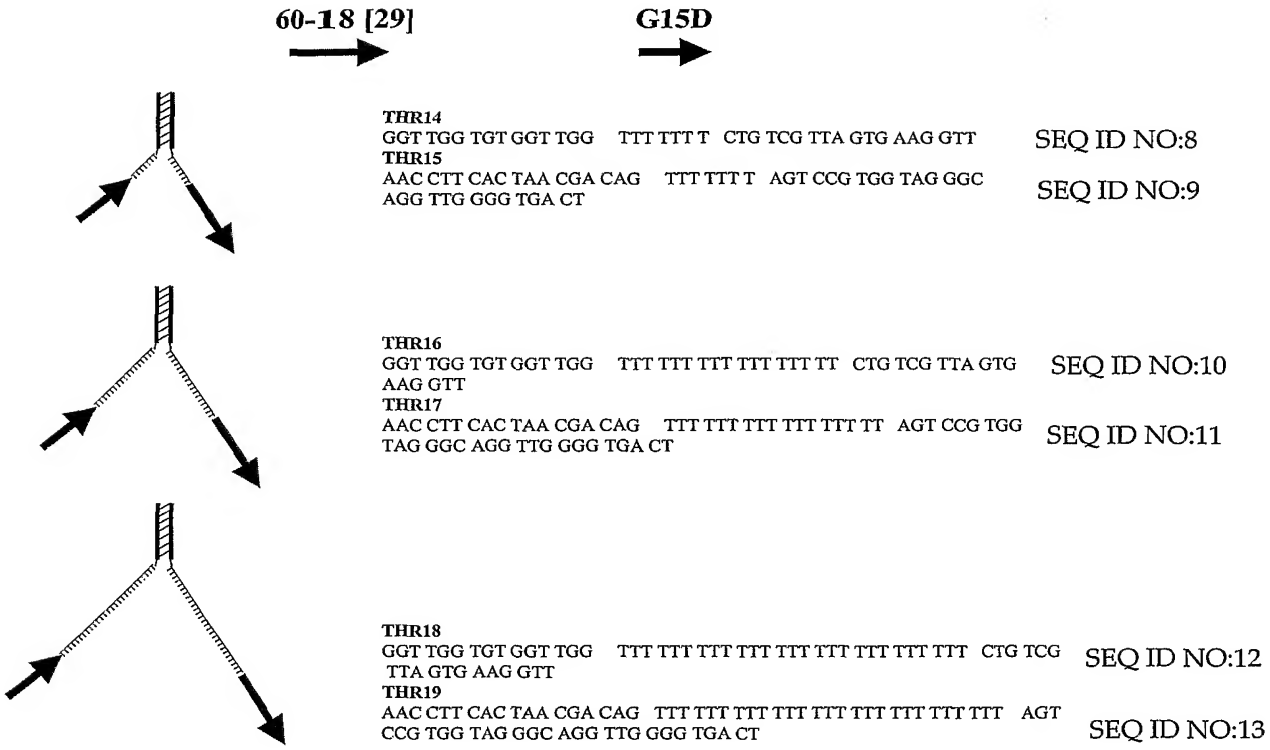

60-18 [29]
G15D
THR14
GGT TGG TGT GGT TGG TTT TTT T CTG TCG TTA GTG AAG GTT
SEQ ID NO:8
THR15
AAC CTT CAC TAA CGA CAG TTT TTT T AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
SEQ ID NO:9
THR16
GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TT CTG TCG TTA GTG AAG GTT
SEQ ID NO:10
THR17
AAC CTT CAC TAA CGA CAG TTT TTT TTT TTT TTT TT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
SEQ ID NO:11
THR18
GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT TTT TTT CTG TCG TTA GTG AAG GTT
SEQ ID NO:12
THR19
AAC CTT CAC TAA CGA CAG TTT TTT TTT TTT TTT TTT TTT TTT TTT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
SEQ ID NO:13

Figure 11

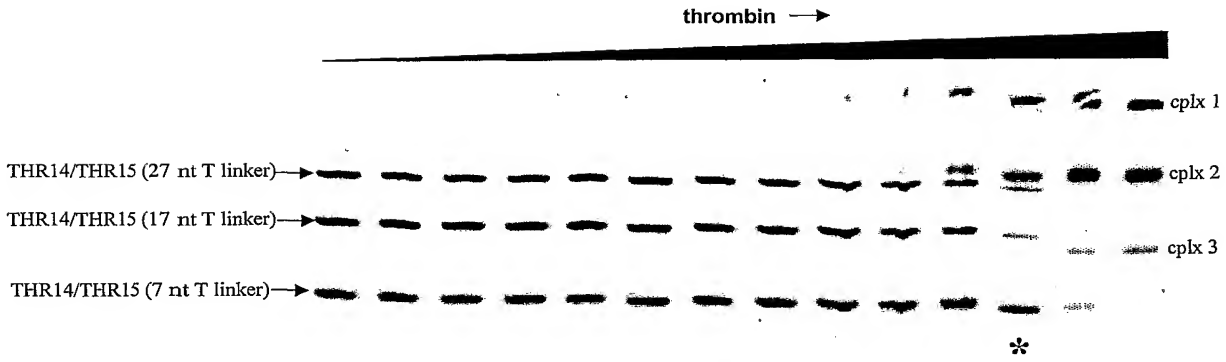

thrombin
cplx 1
cplx 2
cplx 3
THR14/THR15 (27 nt T linker)
THR14/THR15 (17 nt T linker)
THR14/THR15 (7 nt T linker)
*

Figure 12

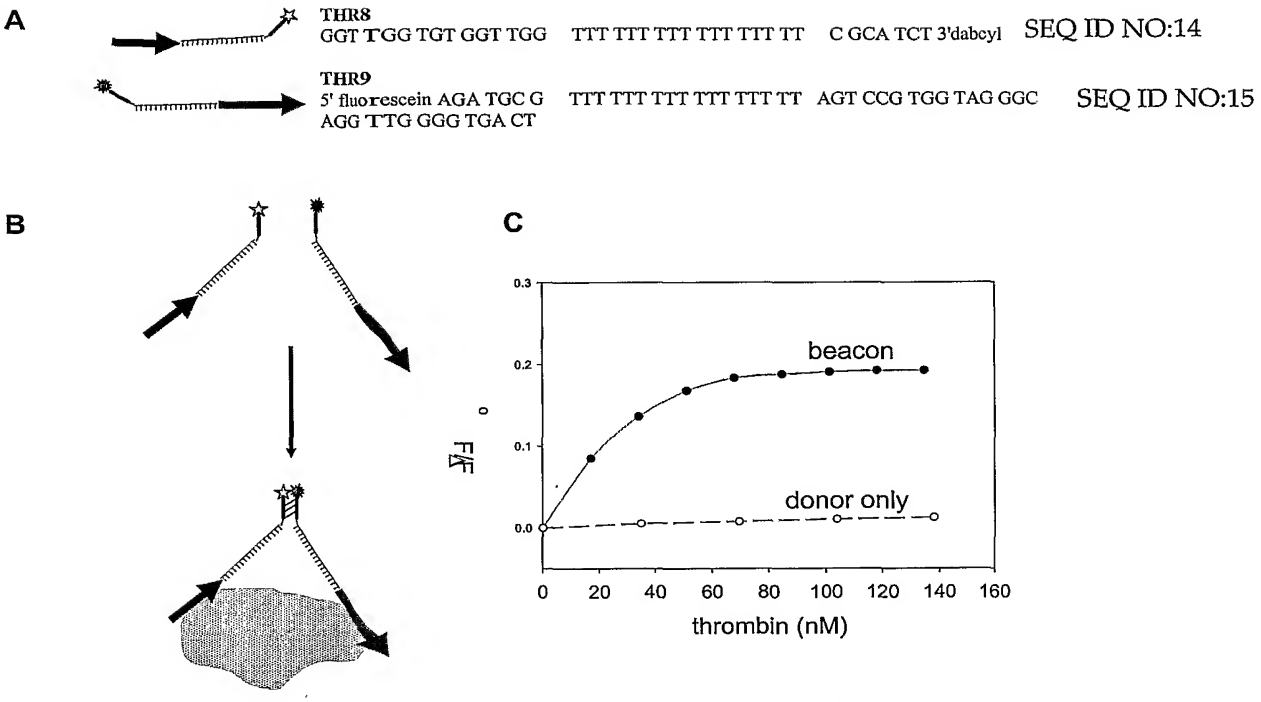

A
THR8
GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TT C GCA TCT 3'dabcyl SEQ ID NO:14
THR9
5' fluorescein AGA TGC G
AGG TTG GGG TGA CT
TTT TTT TTT TTT TTT TT AGT CCG TGG TAG GGC SEQ ID NO:15
B
C
beacon
donor only
F/F0
0.3
0.2
0.1
0.0
0 20 40 60 80 100 120 140 160
thrombin (nM)

Figure 13

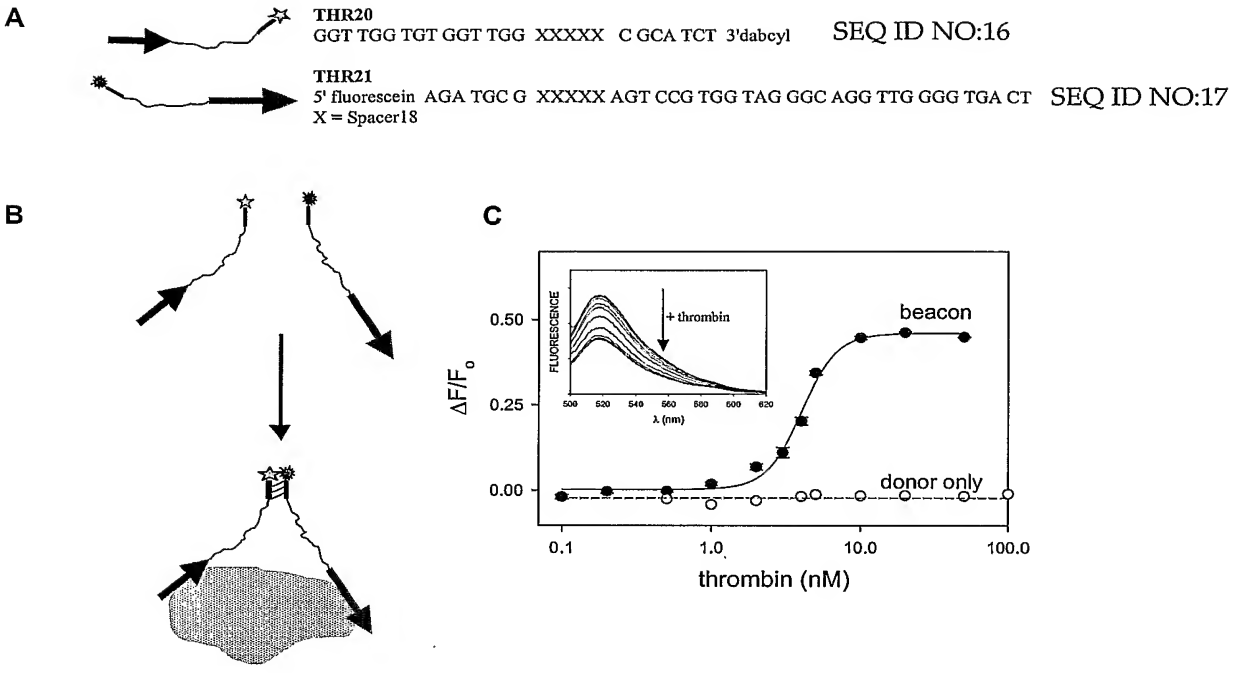

A
THR20
GGT TGG TGT GGT TGG XXXXX C GCA TCT 3'dabcyl
SEQ ID NO:16
THR21
5' fluorescein AGA TGC G XXXXX AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
X = Spacer18
SEQ ID NO:17
B
C
ΔF/F0
0.50
0.25
0.00
FLUORESCENCE
+ thrombin
λ (nm)
beacon
donor only
0.1
1.0
10.0
100.0
thrombin (nM)

Figure 14

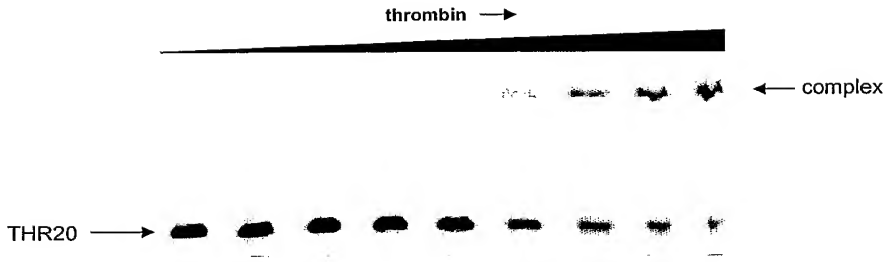


Figure 15

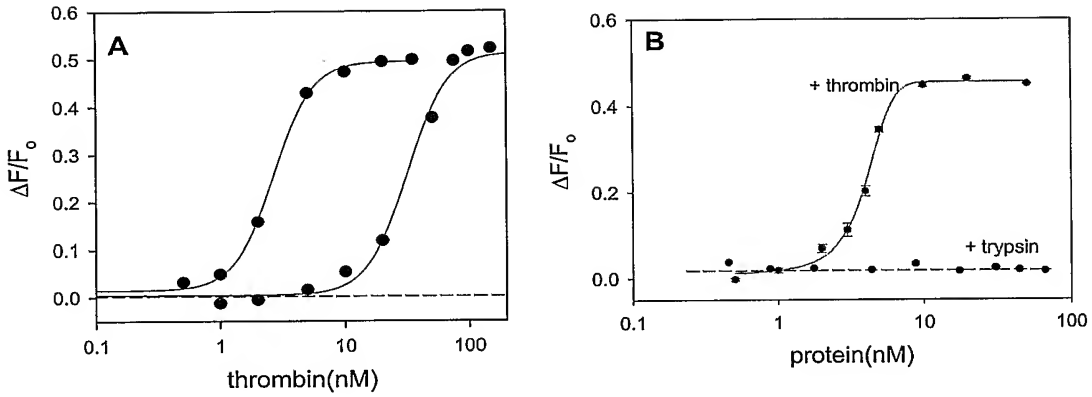

A
B
ΔF/F0
thrombin(nM)
protein(nM)
+ thrombin
+ trypsin
0.1
1
10
100
0.0
0.1
0.2
0.3
0.4
0.5
0.6

Figure 16

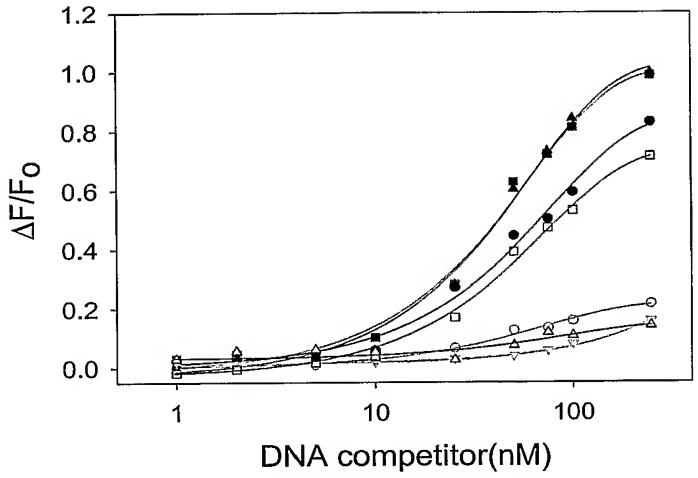


Figure 17

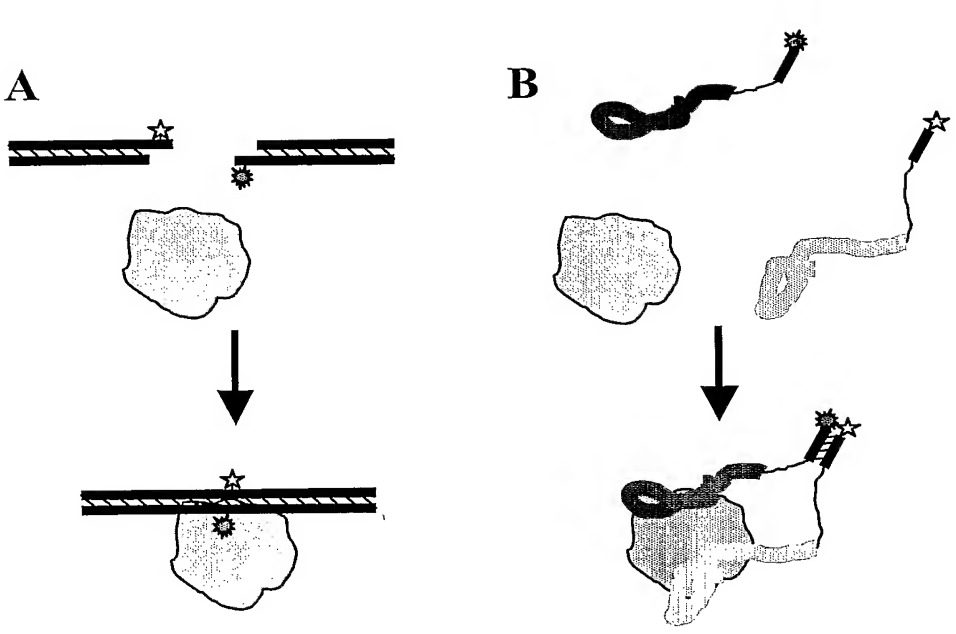


Figure 18

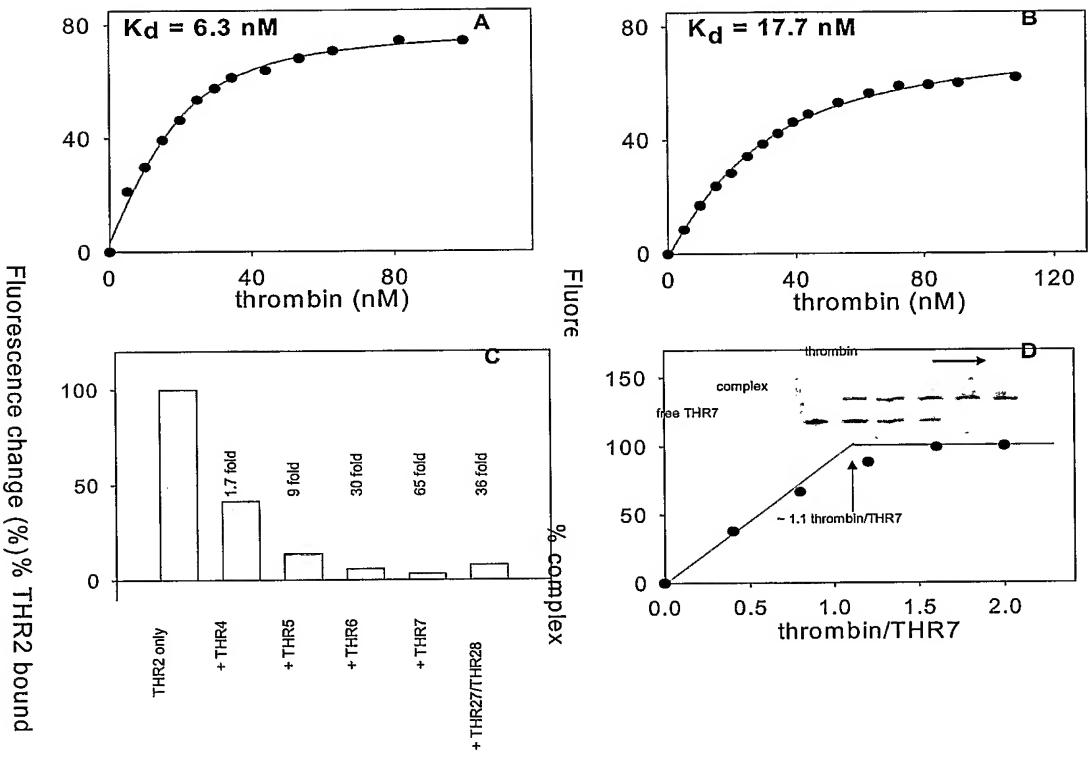

A
Kd = 6.3 nM
thrombin (nM)
B
Kd = 17.7 nM
thrombin (nM)
C
Fluorescence change (%)% THR2 bound
% complex
1.7 fold
9 fold
30 fold
65 fold
36 fold
THR2 only
+ THR4
+ THR5
+ THR6
+ THR7
+ THR27/THR28
D
thrombin
complex
free THR7
~ 1.1 thrombin/THR7
thrombin/THR7

Figure 19

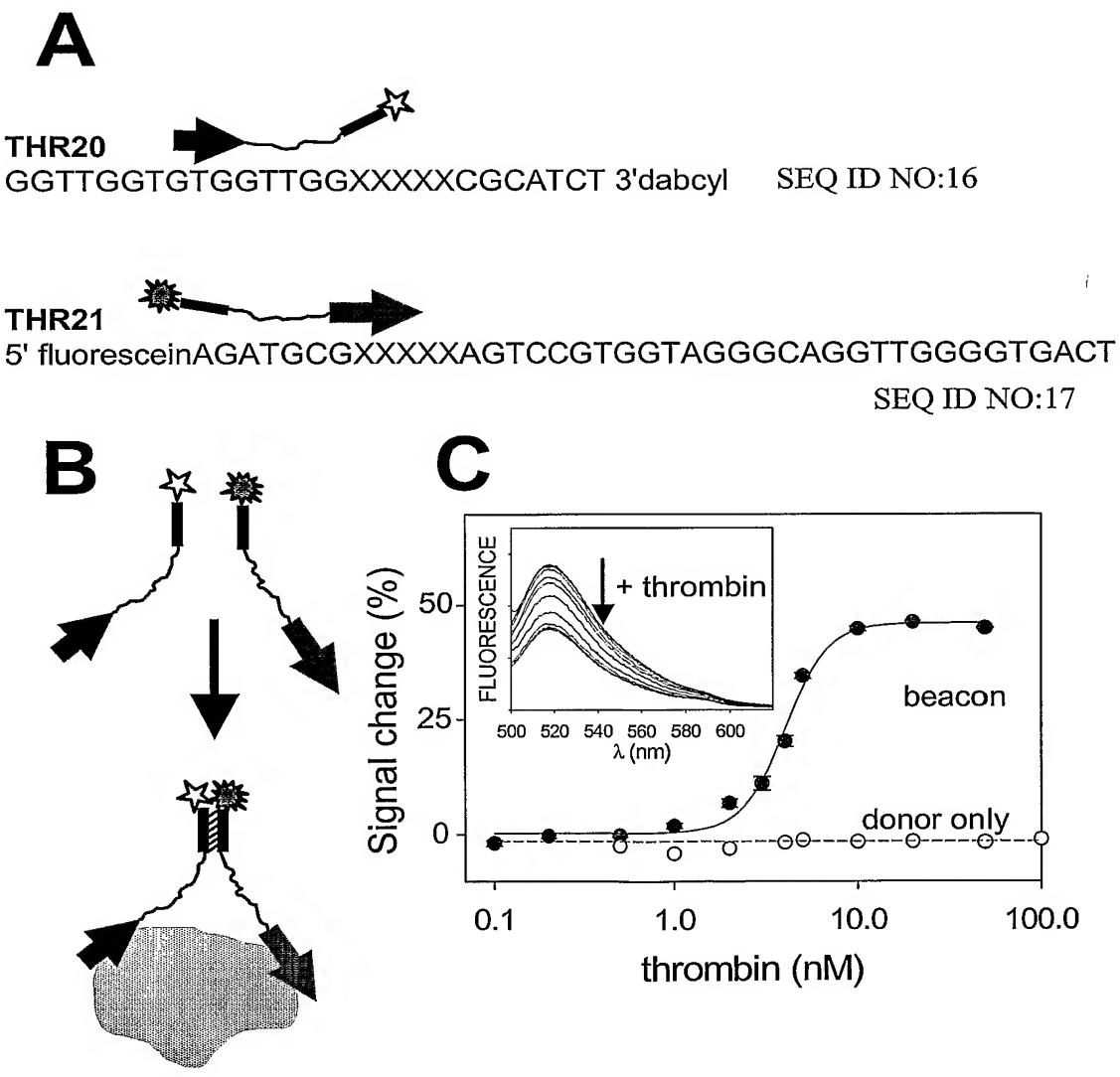

A
THR20
GGTTGGTGTGGTTGGXXXXXCGCATCT 3'dabcyl SEQ ID NO:16
THR21
5' fluoresceinAGATGCGXXXXXAGTCCGTGGTAGGGCAGGTTGGGGTGACT
SEQ ID NO:17
B
C
Signal change (%)
50
25
0
FLUORESCENCE
+ thrombin
500 520 540 560 580 600
λ (nm)
beacon
donor only
0.1
1.0
10.0
100.0
thrombin (nM)

Figure 20

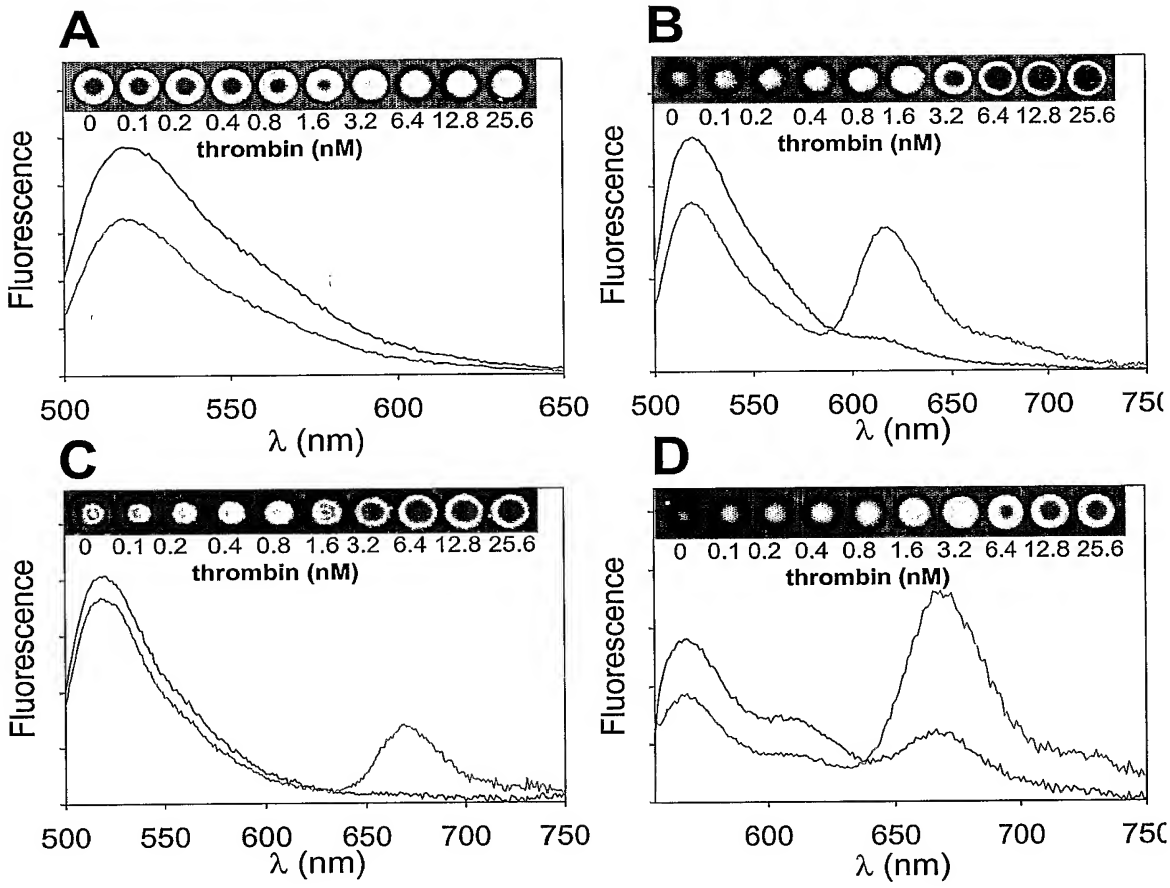

A
0 0.1 0.2 0.4 0.8 1.6 3.2 6.4 12.8 25.6
thrombin (nM)
Fluorescence
500 550 600 650
λ (nm)
B
0 0.1 0.2 0.4 0.8 1.6 3.2 6.4 12.8 25.6
thrombin (nM)
Fluorescence
500 550 600 650 700
λ (nm)
C
0 0.1 0.2 0.4 0.8 1.6 3.2 6.4 12.8 25.6
thrombin (nM)
Fluorescence
500 550 600 650 700 750
λ (nm)
D
0 0.1 0.2 0.4 0.8 1.6 3.2 6.4 12.8 25.6
thrombin (nM)
Fluorescence
600 650 700
λ (nm)

Figure 21

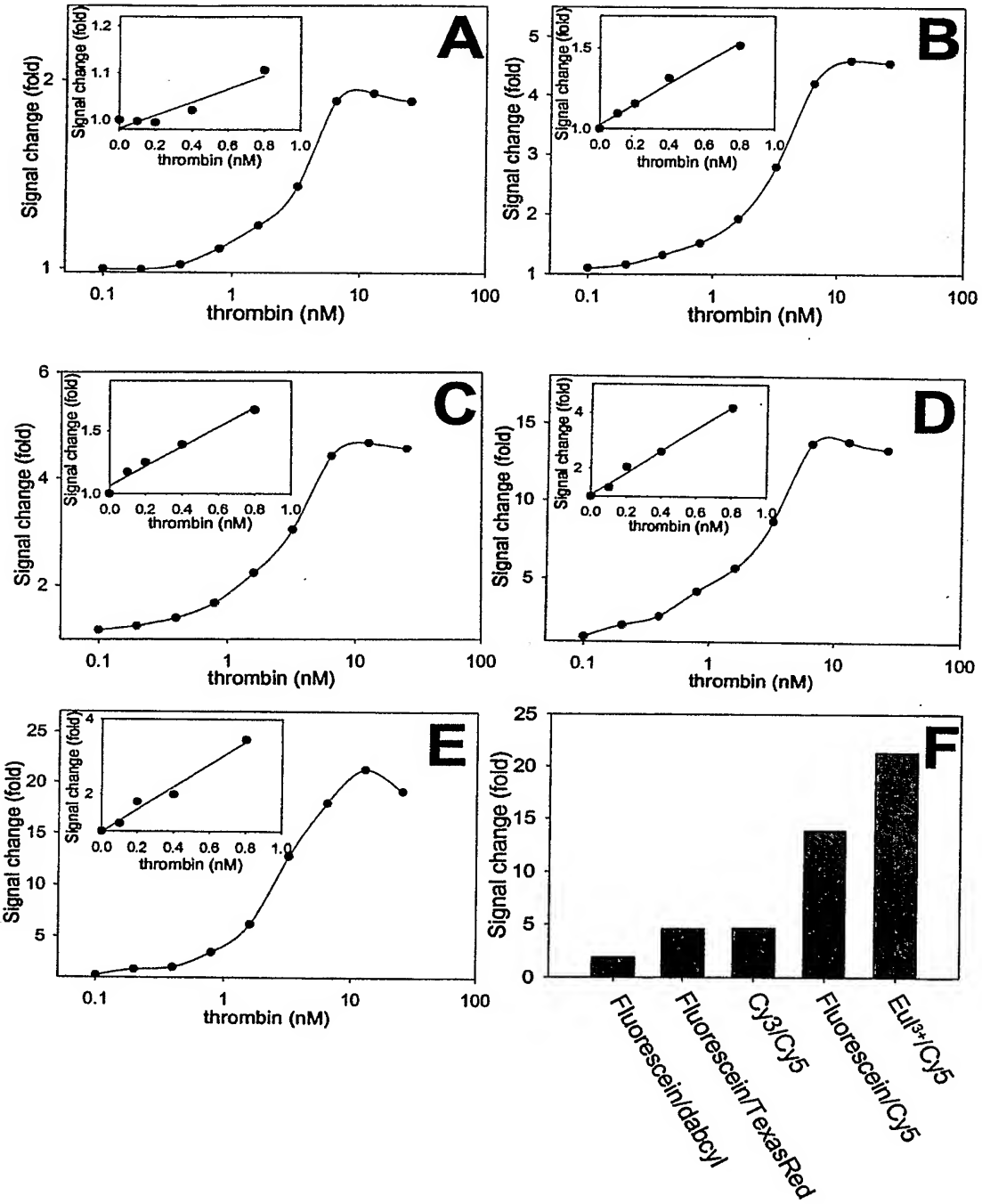

A
B
C
D
E
F
Signal change (fold)
thrombin (nM)
0.1
1
10
100
Fluorescein/dabcyl
Fluorescein/TexasRed
Cy3/Cy5
Fluorescein/Cy5
Eu3+/Cy5

Figure 22

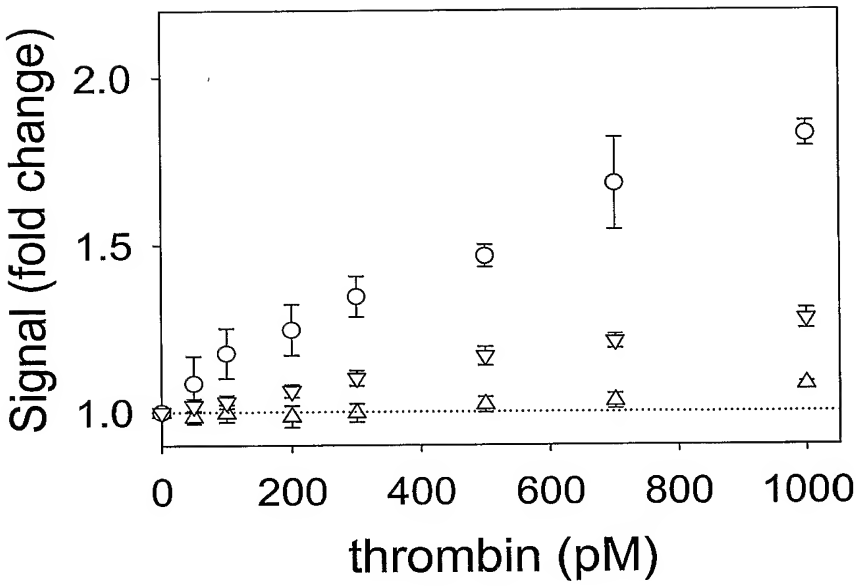


Figure 23

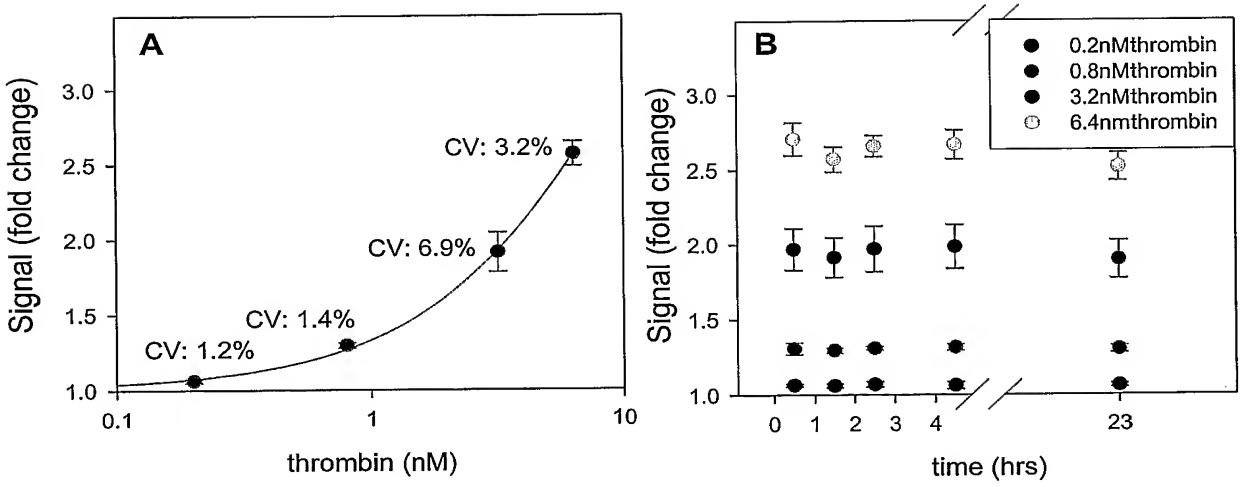

A
Signal (fold change)
3.0
2.5
2.0
1.5
1.0
0.1
1
10
thrombin (nM)
CV: 3.2%
CV: 6.9%
CV: 1.4%
CV: 1.2%
B
Signal (fold change)
0 1 2 3 4
23
time (hrs)
0.2nMthrombin
0.8nMthrombin
3.2nMthrombin
6.4nmthrombin

Figure 24

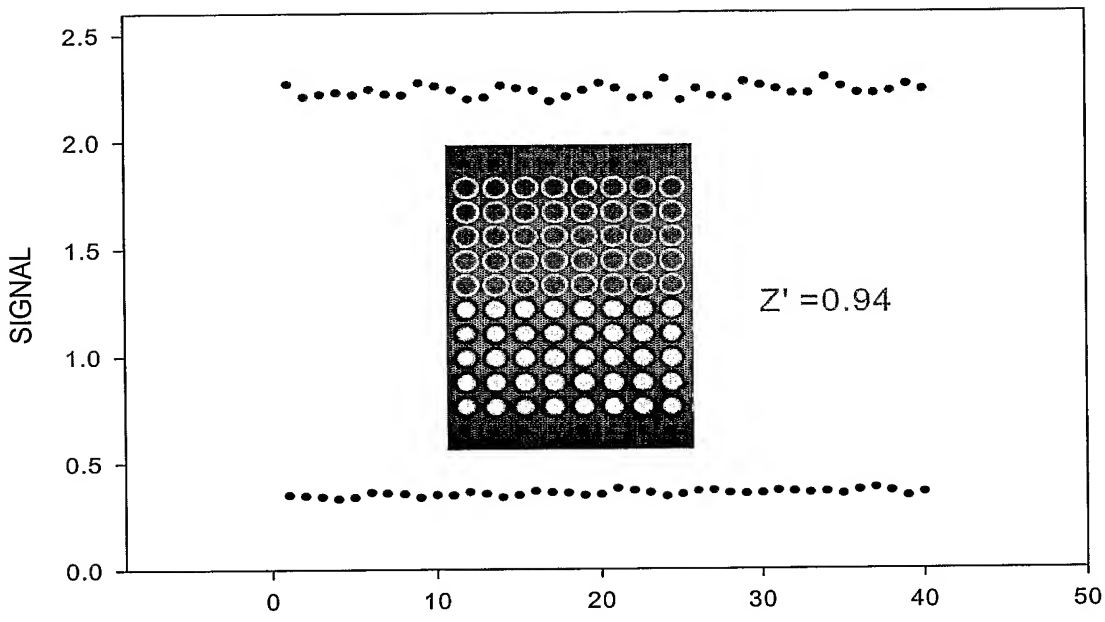
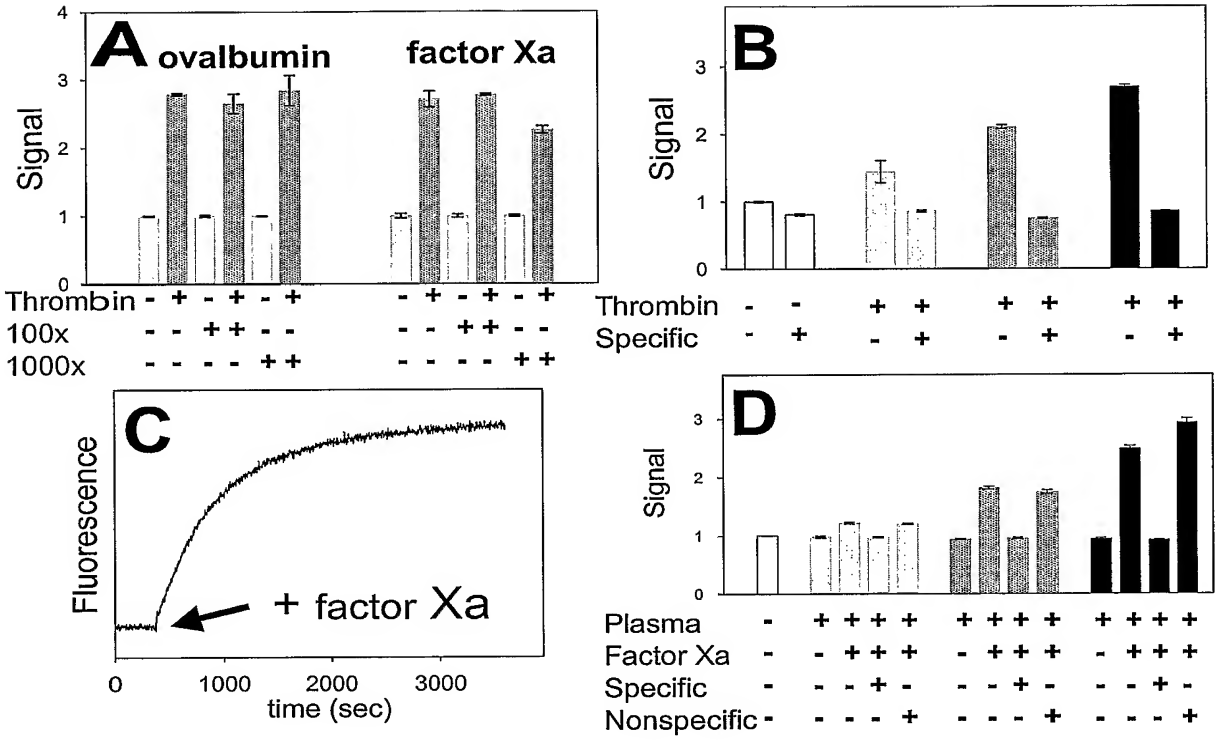


Figure 25


A
ovalbumin
factor Xa
Signal
Thrombin
100x
1000x
B
Signal
Thrombin
Specific
C
Fluorescence
+ factor Xa
time (sec)
D
Signal
Plasma
Factor Xa
Specific
Nonspecific

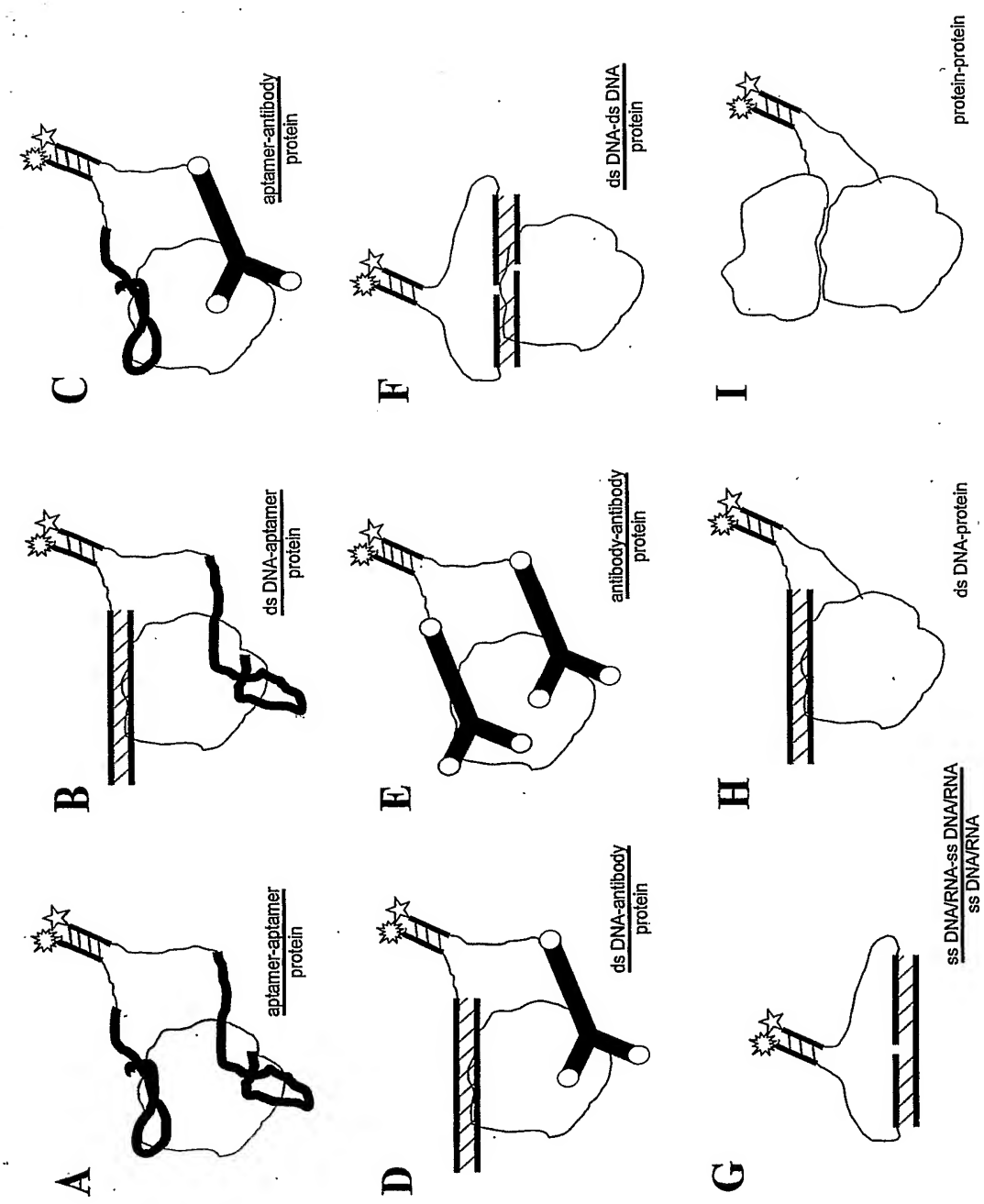

A
aptamer-aptamer
protein
B
ds DNA-aptamer
protein
C
aptamer-antibody
protein
D
ds DNA-antibody
protein
E
antibody-antibody
protein
F
ds DNA-ds DNA
protein
G
ss DNA/RNA-ss DNA/RNA
ss DNA/RNA
H
ds DNA-protein
I
protein-protein

Figure 26

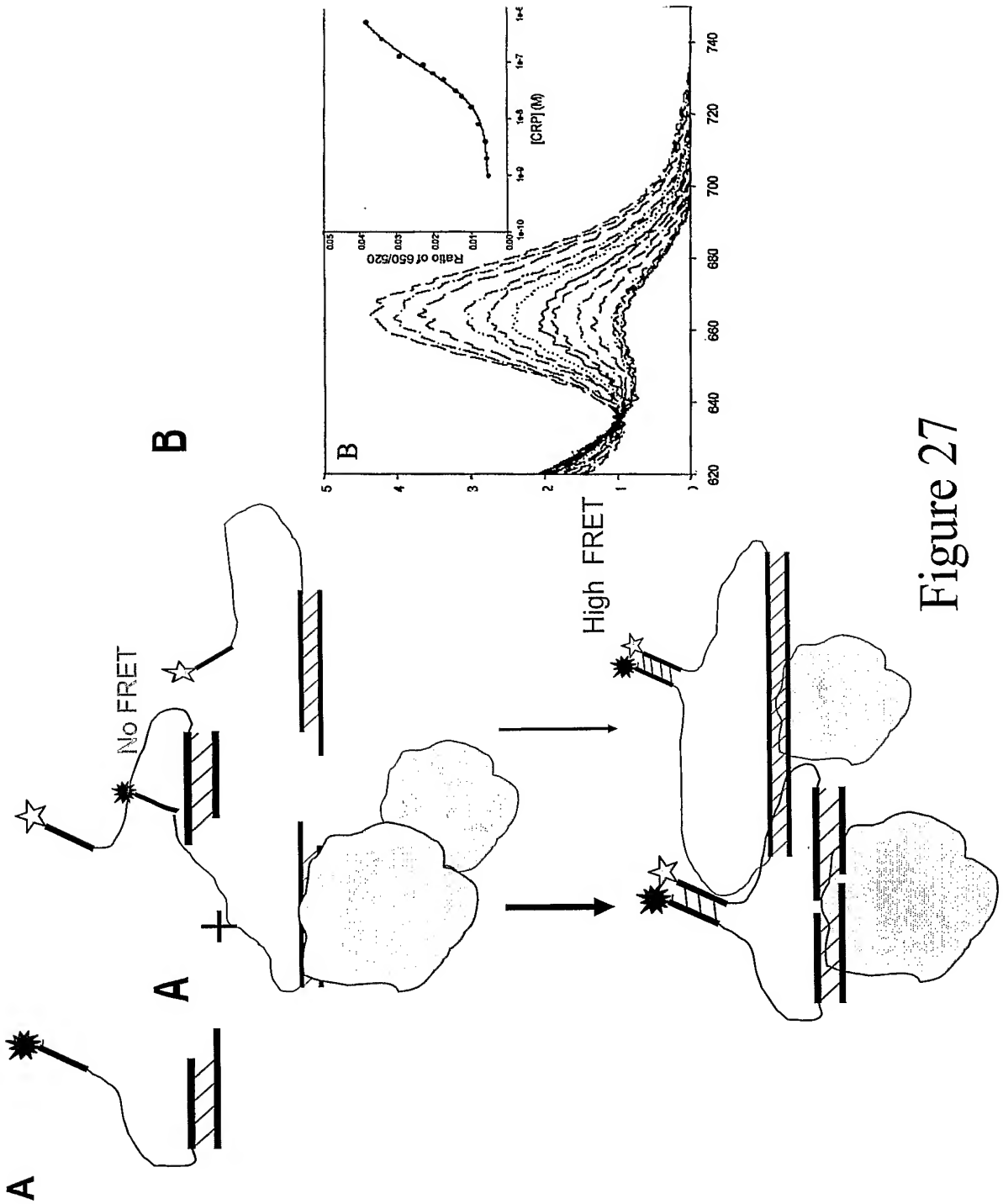


Figure 27

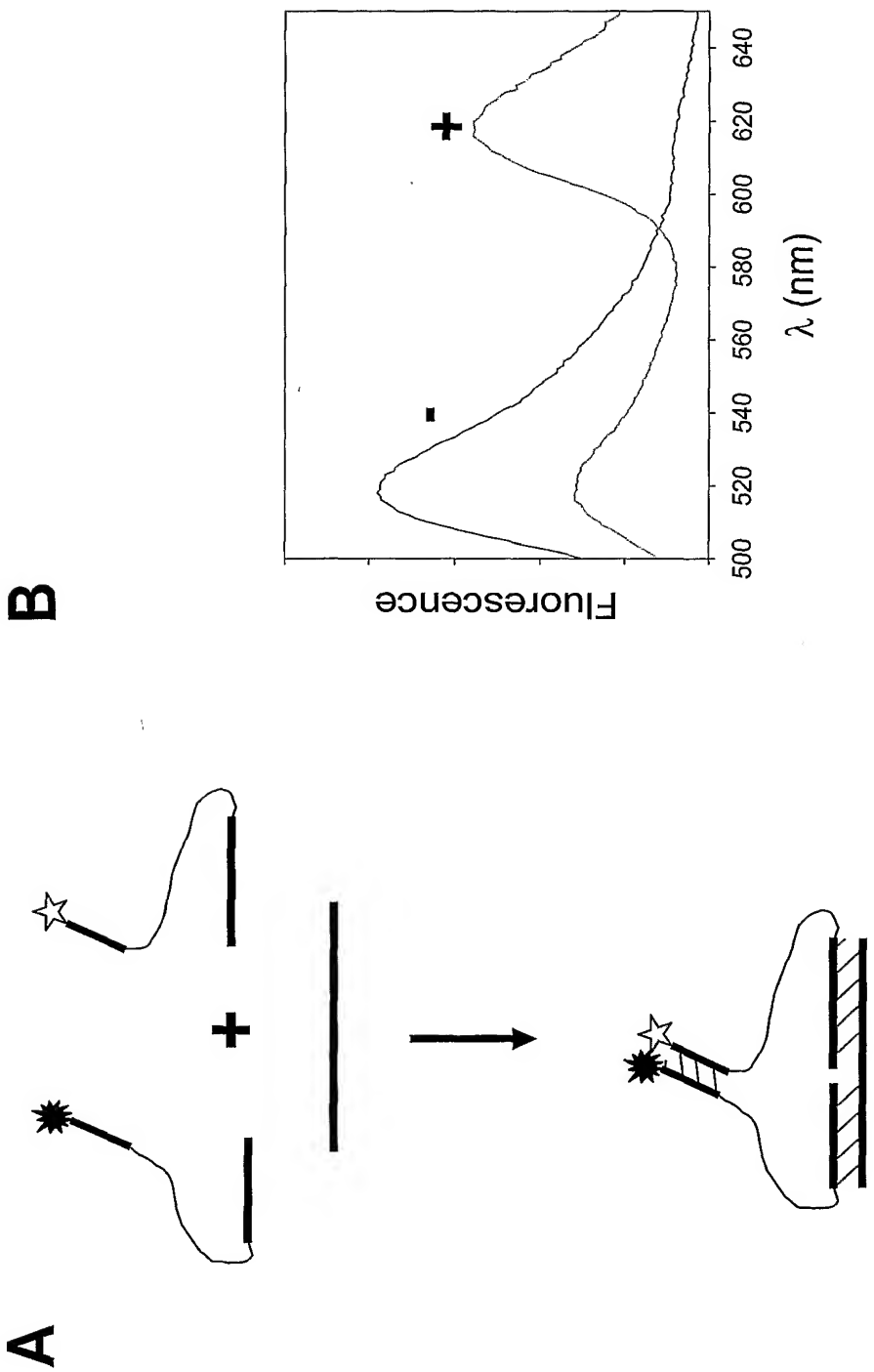
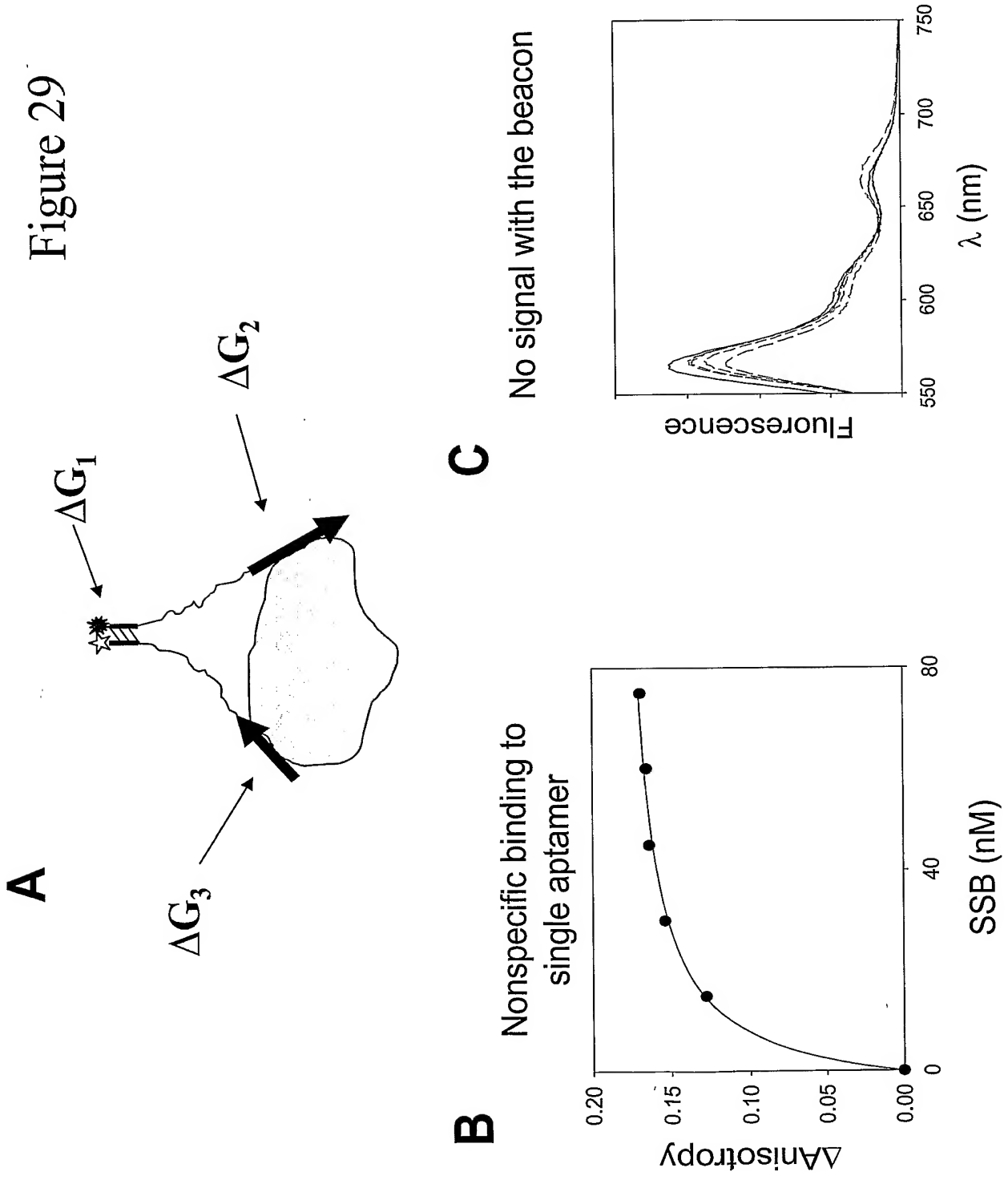


Figure 28

Figure 29


A
ΔG1
ΔG2
ΔG3
B
Nonspecific binding to single aptamer
ΔAnisotropy
0.20
0.15
0.10
0.05
0.00
0
40
80
SSB (nM)
C
No signal with the beacon
Fluorescence
550
600
650
700
750
λ (nm)

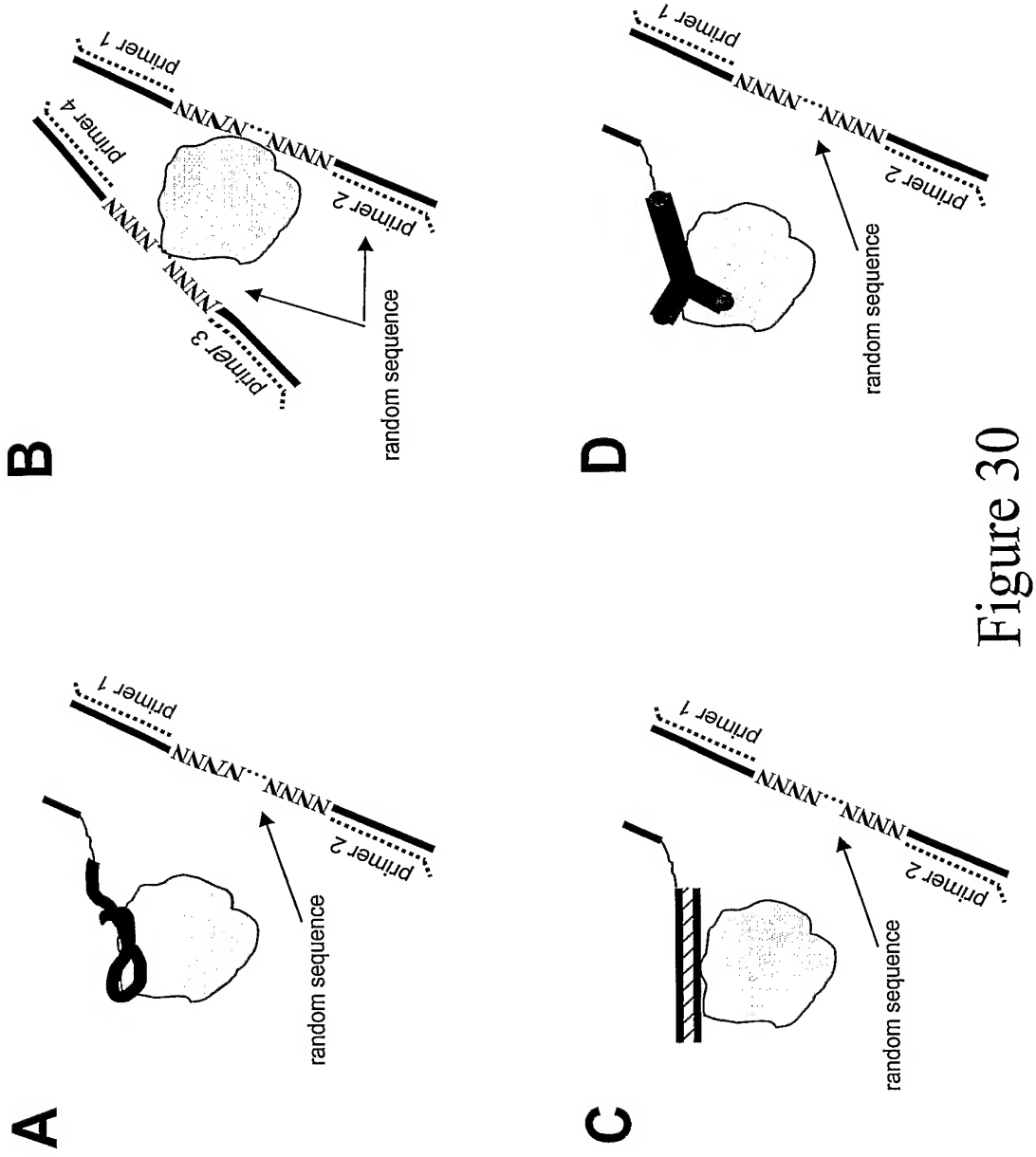

A
primer 1
NNNN...NNNN
primer 2
random sequence
B
primer 1
primer 4
NNNN...NNNN
primer 3
primer 2
random sequence
C
primer 1
NNNN...NNNN
primer 2
random sequence
D
primer 1
NNNN...NNNN
primer 2
random sequence

Figure 30

A
primer 1
NNNN...NNNN
primer 2
random sequence
B
selection
12th
11th
10th
8th
6th
4th
Binding
thrombin(nM)
0
50
100
150
200
250
h
C
Sequence Identifier
clone1 --GGCGGT-ATGGGCATAGCGTAATGGG-AGGTTGGT---------- 33
clone9 --GGCGGT-ATGGGTATAGCGTAATGGG-AGGTTGGT---------- 33
clone7 --GGCGGT-ATGGGCATAGCGTAATGGG-AGGTCTGC---------- 33
clone12 --GCGGNGATAGGTCGCGTAAGTTGGGTAGGGTGG---------- 33
clone2 ----GGATGCGTAATGGTTAGGGTGGGTAGGGTATCC---------- 33
clone3 ----GGATGCGTAATGGTTAGGGTGGGTAGGGTATCC---------- 33
clone4 ----GGATGCGTAATGGTTAGGGTGGGTAGGGTATCC---------- 33
clone8 ----GGATGCGTAATGGTTAGGGTGGGTAGGGTATCC---------- 33
clone23 ----GCATACGTAATGGTCCGGTTGGGGCGGGTATGT---------- 33
clone22 ----GGAGACGTAATGGGTTGGTTGGGAAGNGGATCC---------- 33
clone11 CAGCAGGGAACGGAACGGTTAGGGTGGGTAGGG---------- 33
clone32 ---------GCAGTAGGTACTATATTGGCTAGGGTGGTCTGC---------- 33
clone33 ---------GCAGTAGGTACTATATTGGCTAGGGTGGTCTGC---------- 33
clone29 ---------GCAGTAGGTACTATATTGGCTAGGGTGGTCTGC---------- 33
clone31 ---------GCAGTAGGTACTATATTGGCTAGGGTGGTCTGC---------- 33
clone5 ---------GCAGTAGGTACTATATTGGCTAGGGTGGTCTGC---------- 33
clone6 ---------GCAGTAGGTACTATATTGGCTAGGGTGGTCTGC---------- 33
clone35 ---------GCAGTAGGTACTATGTCGGGTCGGGTGGTCTGC---------- 33
clone10 ----GGGGGTACTAGGTATTAA--TGGGTAGGGTGGTGTT---------- 33
clone34 ----GGGGGTGCTAGGTATTAA--AGGGTAGGGTGGTGT---------- 33
clone20 ----GGGGGTACTAGGTATCAA--TGGGTAGGGTGGTGTT---------- 33
clone21 ----GGGGGTACTAGGTATCAA--TGGGTAGGGTGGTGT---------- 33
clone24 ----GGGGGTACTAGGTATCAA--TGGGTAGGGTGGTGT---------- 33
clone26 ----GGGGGTACTAGGTATCAA--TGGGTAGGGTGGTGT---------- 33
clone13 ---------------------CAGGATGGGTAGGGTGGTCAGCGAAGCAGTAGG 33
clone15 ---------------------CAGGATGGGTAGGGTGGTCAGCGAAGCAGTAGG 33
clone16 ---------------------CAGGATGGGTAGGGTGGTCAGCGAAGCAGTAGG 33
clone19 ---------------------CAGGATGGGTAGGGTGGTCAGCGAAGCAGTAGG 33
clone25 ----------GAGGGGACTTAGGATGGGTAGGGTGGTAGGCCC---------- 33
clone17 ---GGCGAGAGCAGCGTGATAGGGTGGGTAGGGTGG---------- 33
clone14 ---CAACGGTTGGGTGAACTGTAGTGGCTTGGGGTG---------- 33
clone27 ---GGTCGGGGCATAGT--AATGCTGGATTGGGCAGCT---------- 33
clone28 --GGGTAGGAGCAGTAC--A-CGCTGGAATGGGTCACT---------- 33
clone30 --GGGTAGGGTGACAGG--GAGGACGGAATGGGC-ACT---------- 33
clone18 -----CAGGGTCAGGGCTAGATGATGCGATTAACCATG---------- 33
ruler 1.....10.....20.....30.....40.....50..

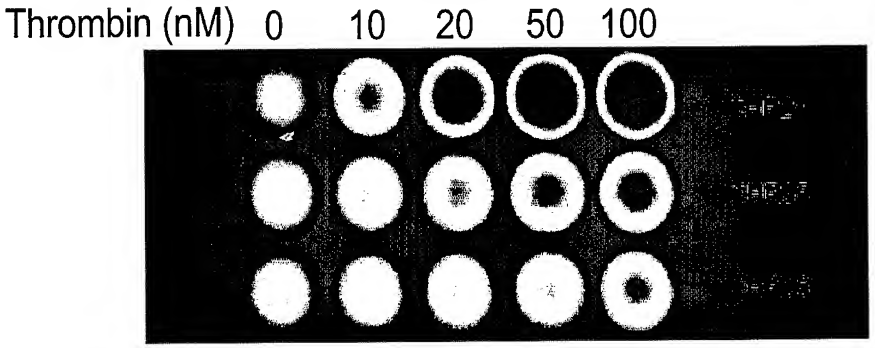
Figure 31

clones 20-26

5'fluoresceinAGATGCGXXXXX AG GTT GGG GGT ACT AGG TAT CAA TGG GTA GGG TGG TGT AACGC THR3 5

5'fluoresceinAGATGCGXXXXXA GTG AAG GTT GGG GGT ACT AGG TAT CAA TGG GTA GGG TGG TGT AAC GCC ATA T THR3 6

A



B

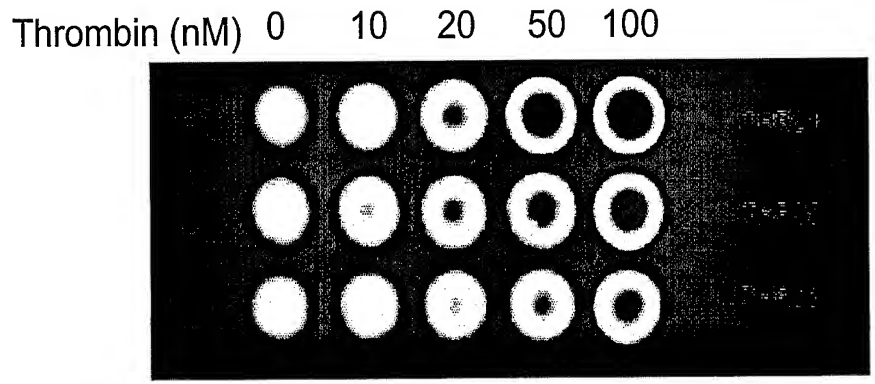


Figure 32

A
primer 1
primer 4
primer 3
primer 2
NNNN
random sequence
B
selection
13th
14th
12th
11th
10th
8th
Binding
0
50
100
150
200
250
thrombin(nM)
C
Sequence Identifier
clone1-1 --GGGTAGGGTGGTTGTAATAGGG--ATTGCGAT----- 80 30
clone1-2 --GGGTAGGGTGGTTGTAATAGGG--ATTGCGAT----- 81 30
clone1-4 --GGGTAGGGTGGTTGTAATAGGG--ATTGCGAT----- 83 30
clone1-5 --GGGTAGGGTGGTTGTAATAGGG--ATTGCGAT----- 84 30
clone1-9 --GGGTAGGGTGGTTGTAATAGGG--ATTGCGAT----- 88 30
clone2-4 ---GGTAGGGTGGTTAAAATAGGGGAATGGCAG------ 93 30
clone2-7 ---GGTAGGGTGGTTAAAATAGGGGAATGGCAG------ 96 30
clone2-10 ---GGTAGGGTGGTTAAAATAGGGGAATGGCAG------ 99 30
clone2-8 -----TAGGATGGGTAGGGTGGTCCCAGGAATGGC---- 97 30
clone2-9 -----TAGGATGGGTAGGGTGGCCCCAGGAATGGC---- 98 30
clone2-12 -----TAGGATGGGTAGGGTGGTCCCAGGAATGGC---- 101 30
clone2-14 -----TAGGATGGGTAGGGTGGTCCCAGGAATGGC---- 103 30
clone1-8 ACGCGTAGGATGGGTAGGGTGGTCGCGTTA---------- 87 30
clone2-2 -----TAGGGTGGGTAGGGTGGTCAACTATGGGGG----- 91 30
clone1-7 ---AATGGGAGGTTGGGGGTGCGGGAGAGTGGT------ 86 30
clone2-5 ---CACAGAAGGGCGAGCGCTGAGCATAGTGC-------- 94 30
clone2-13 -------GGAGATGCAGGTACTGAGTAGGGAGTGTGC--- 102 30
clone2-3 -------GGGTGGCTGGTCAAGGAG-ATAGTACGATGC-- 29 30
clone2-11 -------GATG--TGGCCCAGAAGCATAACACGACGTAC 100 30
clone2-1 --AAGGCCGCCATCTGGGTCCGACGAGTACCA-------- 90 30
clone1-10 -------GGGCGAAGGTACGAAGACGGATGCACCGTGC--- 89 30
clone1-6 -------GGTGTGGGTGG-TTATTGGTGTAGAGCGGGT--- 85 30
clone1-3 -------GGCACAACCCGATATGGCTATGAATCTGCC---- 82 30
clone2-6 ---CCAACGACACATAGGGTACACGCCGCCTCC------- 95 30
ruler 1.....10.....20.....30.....40

Figure 33

A
primer 1
NNNN...NNNN
primer 2
random sequence
B
selection
11th
10th
9th
Binding
CRP(nM)
0
50
100
150
200
250
C
Sequence Identifier
clone1 104
clone23 126
clone3 106
clone17 120
clone15 118
clone7 110
clone20 123
clone5 108
clone10 113
clone13 116
clone2 105
clone6 109
clone9 112
clone16 119
clone21 124
clone4 107
clone22 125
clone8 111
clone14 117
clone18 121
clone12 115
clone19 122
clone11 114
ruler 1
10
20
30
40
50
33

Figure 34

SEQUENCE LISTING

<110> Heyduk, Tomasz
Heyduk, Ewa
Knoll, Eric

<120> BIOSENSORS FOR DETECTING MACROMOLECULES AND OTHER ANALYTES

<130> SLU 03-015 PCT

<150> US 60/529,076
<151> 2003-12-12

<160> 126

<170> PatentIn version 3.3

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<211> 15
<212> DNA
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<220>
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ggttggtgtg gttgg 15

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<220>
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agtccgtggt agggcaggtt ggggtgact 29

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<212> DNA
<213> Artificial

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<223> based on mammalian sequence

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ggttggtgtg gttgg 15

<210> 5
<211> 49
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
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<223> n is a, c, g, t or u

<400> 5
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<210> 6
<211> 54
<212> DNA
<213> Artificial

<220>
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<223> n is a, c, g, t or u

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<210> 7
<211> 54
<212> DNA
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<223> based on mammailian sequence

<220>
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<223> n is a, c, g, t or u

<400> 7
ggttggtgtg gttggnnnnn nnnnnagtcc gtggtagggc aggttggggt gact 54

<210> 8
<211> 40

<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 8
ggttggtgtg gttggttttt ttctgtcgtt agtgaaggtt 40

<210> 9
<211> 54
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 9
aaccttcact aacgacagtt tttttagtcc gtggtagggc aggttggggt gact 54

<210> 10
<211> 50
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 10
ggttggtgtg gttggttttt ttttttttt ttctgtcgtt agtgaaggtt 50

<210> 11
<211> 64
<212> DNA
<213> Artificial

<220>
<223> based on mammalian

<400> 11
aaccttcact aacgacagtt ttttttttt tttttagtcc gtggtagggc aggttggggt 60

gact 64

<210> 12
<211> 60
<212> DNA
<213> Artificial

<220>
<223> based on mammailian sequence

<400> 12
ggttggtgtg gttggttttt ttttttttt ttttttttt ttctgtcgtt agtgaaggtt 60

<210> 13

<211> 74
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 13
aaccttcact aacgacagtt ttttttttt ttttttttt tttttagtcc gtggtagggc 60

aggttggggt gact 74

<210> 14
<211> 39
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

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ggttggtgtg gttggttttt ttttttttt ttcgcatct 39

<210> 15
<211> 53
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 15
agatgcgttt ttttttttt ttttagtccg tggtagggca ggttggggtg act 53

<210> 16
<211> 27
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<213> Artificial

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<220>
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ggttggtgtg gttggnnnnn cgcatct 27

<210> 17
<211> 41
<212> DNA
<213> Artificial

<220>

<223> based on mammalian sequence

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<222> (8)..(12)
<223> n is a, c, g, t or u

<400> 17
agatgcgnnn nnagtccgtg gtagggcagg ttggggtgac t 41

<210> 18
<211> 28
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
<222> (16)..(20)
<223> n is a, c, g, t or u

<400> 18
ggttggtgtg gttggnnnnn tcgcatct 28

<210> 19
<211> 41
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
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<223> n is a, c, g, t or u

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agatgcgnnn nnagtccgtg gtagggcagg ttggggtgac t 41

<210> 20
<211> 69
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
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<222> (19)..(51)
<223> n is a, c, g, t or u

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tcacagacg 69

<210> 21
<211> 18
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 21
ctgtcgttag tgaaggtt 18

<210> 22
<211> 18
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 22
cgtctgtgat atggcgtt 18

<210> 23
<211> 22

<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

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<223> n is a, c, g, t or u

<400> 23
ggttggtgtg gttggnngac ag 22

<210> 24
<211> 27
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature

<222> (16)..(20)
<223> n is a, c, g, t or u

<400> 24
ggttggtgtg gttggnnnnn acgacag 27

<210> 25
<211> 24
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
<222> (13)..(17)
<223> n is a, c, g, t or u

<400> 25
gaacgagagt gcnnnnncgc atct 24

<210> 26
<211> 24
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
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<222> (8)..(12)
<223> n is a, c, g, t or u

<400> 26
agatgcgnnn nnttgaactg gacc 24

<210> 27
<211> 26
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequnece

<400> 27
ggtccagttc aattgcactc tcgttc 26

<210> 28
<211> 28
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<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
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<223> n is a, c, g, t or u

<400> 28
ggttggtgtg gttggnnnnn aacgacag 28

<210> 29
<211> 82
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
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<222> (9)..(13)
<223> n is a, c, g, t or u

<220>
<221> misc_feature
<222> (32)..(64)
<223> n is a, c, g, t or u

<400> 29
ctgtcgttnn nnnttgagtc agcgtcgagc annnnnnnnn nnnnnnnnnn nnnnnnnnnn 60

nnnnttcact gtgctgcggc ta 82

<210> 30
<211> 31
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
<222> (9)..(13)
<223> n is a, c, g, t or u

<400> 30
ctgtcgttnn nnnttgagtc agcgtcgagc a 31

<210> 31
<211> 18
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 31
tagccgcagc acagtgaa 18

<210> 32
<211> 66
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
<222> (19)..(48)
<223> n is a, c, g, t or u

<400> 32
cacctgatcg ctcctcgtnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnca ggatgcacag 60

gcacaa 66

<210> 33
<211> 66
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
<222> (19)..(48)
<223> n is a, c, g, t or u

<400> 33
agccgccatt ccatagtgnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnca ggatgccgat 60

caggtg 66

<210> 34
<211> 18
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 34
cacctgatcg ctcctcgt 18

<210> 35
<211> 18

<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 35
ttgtgcctgt gcatcctg 18

<210> 36
<211> 18
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 36
agccgccatt ccatagtg 18

<210> 37
<211> 18
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 37
cacctgatcg gcatcctg 18

<210> 38
<211> 55
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
<222> (8)..(12)
<223> n is a, c, g, t or u

<400> 38
agatgcgnnn nnaggttggg ggtactaggt atcaatgggt agggtggtgt aacgc 55

<210> 39
<211> 65
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<220>
<221> misc_feature
<222> (8)..(12)
<223> n is a, c, g, t or u

<400> 39
agatgcgnnn nnagtgaagg ttgggggtac taggtatcaa tgggtagggt ggtgtaacgc 60

catat 65

<210> 40
<211> 50
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

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<222> (39)..(43)
<223> n is a, c, g, t or u

<400> 40
aacgcaataa atgtgaagta gatcacattt taggcaccnn nnngatggct 50

<210> 41
<211> 69
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

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<223> n is a, c, g, t or u

<400> 41
agccatctaa ctattcccnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn ngagcgagaa 60

attctaggt 69

<210> 42
<211> 38
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 42
ggtgcctaaa atgtgatcta cttcacattt attgcgtt 38

<210> 43
<211> 18
<212> DNA
<213> Artificial

<220>
<223> baased on mammalian sequence

<400> 43
agccatctaa ctattccc 18

<210> 44
<211> 18
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 44
acctagaatt tctcgctc 18

<210> 45
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 45
ggcggtatgg gcatagcgta atgggaggtt ggt 33

<210> 46
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 46
ggatgcgtaa tggttagggt gggtagggta tcc 33

<210> 47
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 47
ggatgcgtaa tggttagggt gggtagggta tcc 33

<210> 48

<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 48
ggatgcgtaa tggttagggt gggtagggta tcc 33

<210> 49
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 49
gcagtaggta ctatattggc tagggtggtc tgc 33

<210> 50
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 50
gcagtaggta ctatattggc tagggtggtc tgc 33

<210> 51
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammlian sequence

<400> 51
ggcggtatgg gcatagcgta atgggaggtc tgc 33

<210> 52
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 52
ggatgcgtaa tggttagggt gggtagggta tcc 33

<210> 53
<211> 33

<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 53
ggcggtatgg gtatagcgta atgggaggtt ggt 33

<210> 54
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 54
gggggtacta ggtattaatg ggtagggtgg tgt 33

<210> 55
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequences

<400> 55
cagcagggaa cggaacggtt agggtgggta ggg 33

<210> 56
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequences

<220>
<221> misc_feature
<222> (5)..(5)
<223> n is a, c, g, t or u

<400> 56
gcggngatag gtcgcgtaag ttgggtaggg tgg 33

<210> 57
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequences

<400> 57

caggatgggt agggtggtca gcgaagcagt agg 33

<210> 58
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 58
caacggttgg gtgaactgta gtggcttggg gtg 33

<210> 59
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequences

<400> 59
caggatgggt agggtggtca gcgaagcagt agg 33

<210> 60
<211> 32
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 60
caggatgggt agggtggtca gcgaagcagt ag 32

<210> 61
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 61
ggcgagagca gcgtgatagg gtgggtaggg tgg 33

<210> 62
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequences

<400> 62
cagggtcagg gctagatgat gcgattaacc atg 33

<210> 63
<211> 33
<212> DNA
<213> Artificial

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<223> based on mammalian sequence

<400> 63
caggatgggt agggtggtca gcgaagcagt agg 33

<210> 64
<211> 33
<212> DNA
<213> Artificial

<220>
<223> based on mammalian sequence

<400> 64
gggggtacta ggtatcaatg ggtagggtgg tgt 33

<210> 65
<211> 33
<212> DNA
<213> Artificial

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